STUDIES ON CELL FUSION

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The cells of a rat myogenic cell line, L_5, have been cultured. It was found that these cells could be switched from proliferation to differentiation, if the concentration of serum in the medium was lowered to 1-2% from the 10% level normally used to maintain the cells. A number of clones of 'failed myoblasts', which have lost the capacity to differentiate, have been isolated.

L_5 cells have been shown to be capable at one time of going through a mitosis or of fusing into a myotube without going through a round of DNA synthesis. The implications of this finding for the 'quantal mitosis' theory are discussed.

Artificial syncytia have been made from L_5 myoblasts by Sendai virus-induced cell fusion. These syncytia do not exhibit the normal properties of differentiated syncytial myotubes.

The capacity of mononucleate myoblast x fibroblast hybrid cells to undergo myogenesis has been studied. Myotube formation and the attainment of elevated levels of creatine phosphokinase are suppressed in these cells.

Chicken erythrocyte nuclei have been introduced into myoblasts and myotubes in cultures of L_5 cells. Chicken muscle specific antigens have been found

subsequently to appear in some of the myotubes in these cultures.

Nuclei have been identified on a species specific basis in rat/mouse heterokaryotic myotubes.

CONTENTS

CHAPTER VII : Identification of nuclei on a species specific basis in myogenic cultures by *in situ* hybridisation

CHAPTER I

Introduction

Inheritance of the epigenetic state

One of the remarkable things about the differentiation of cells is that in many cases once a cell has been induced to take up a differentiated state it can replicate and still apparently 'remember' its particular state of differentiation. Pigment cells provide a good example of this phenomenon. These cells arise as a migratory cell type (in the neural crest) and move through the embryo to their final positions. The melanocytes in, for instance, the skin, are descended from cells which have come from the neural crest and which therefore appear to remember the inductive effects conferred on them there. This inheritance of the epigenetic state becomes even more apparent in the case of the melanoma. When a pigment cell turns neoplastic the cancer is liable to metastasise all over the body. Thousands of melanistic colonies can appear, far from their original position and cellular microenvironment and yet maintaining their original state of differentiation.

It is possible to cultivate cells in dishes and they can be shown outside the body to maintain properties characteristic of the tissue from which they were explanted: melanoblasts produce pigment (Cahn & Cahn 1966), myoblasts make multinucleate muscle syncytia

containing the contractile proteins (Konigsberg 1961) and neuroblasts develop a number of neurological properties (Augusti-Tocco & Sato 1969). The fact that different cell types can reproduce themselves under standard conditions and that they do not interconvert in vitro - myoblasts do not suddenly begin to make melanin - suggests that they are not being continuously induced by the complex culture medium to their particular state of differentiation. Is it possible that cells 'remember' their cell type in vitro by, for instance, simply continuing to translate messenger RNAs already present in the cells at the time that they are explanted? That this is not the case is shown by the existence of differentiating cell lines. Cells taken from the body will normally only undergo a limited number of divisions in culture, but the cells of some species occasionally give rise for unknown reasons to clones which have overcome this ageing block and are apparently immortal. These cells do not necessarily lose their state of differentiation when they overcome the ageing block and many immortal cell lines continuously show differentiated properties in their subclones after a number of divisions in culture sufficient to have diluted out all the molecules originally present. In some way the differentiated state is self-replicating.

This thesis deals with experiments designed to

elucidate the way in which the epigenetic state is inherited and expressed in one particular cell type, the myoblast.

Reasons for studying myogenic cells

There are several reasons why myogenesis is a particularly suitable system for the study of cell differentiation. First of all myoblasts can be cultured *in vitro* and external factors which influence their behaviour can therefore be experimentally manipulated. Another special feature of myogenesis is that the switch of the epigenetically determined precursor cell from the proliferative phase to the non-mitotic terminally differentiated state, which is characteristic of several differentiating cell types (Green and Todaro 1967), is particularly marked and is accompanied by the overt event of myotube formation. The differentiated myotube itself has many distinctive features which have been well studied and which can be detected by a variety of techniques. The final reason for studying myogenesis is the availability of immortal myogenic cell lines. The interpretation of observations made on differentiation *in vivo* or on cultures of cells which are newly explanted is complicated by the fact that tissues always contain a mixture of cell types. Clones of cells derived from myogenic cell lines provide pure cultures of cells which are all epigenetically determined in the same way.

Basic features of myogenesis in vitro

Overt changes in differentiation

When the cells of embryonic vertebrate muscle are disaggregated and put into appropriate culture conditions they will undergo differentiation *in vitro*. Most of the cells which grow in a primary culture of newborn rat muscle are refractile, bipolar, spindle-shaped myoblasts (Yaffe 1968). These cells will multiply in culture but after a period will align together and fuse to make long syncytial muscle straps or 'myotubes' which may begin to twitch in the dish, (Yaffe 1971). The myoblasts of chickens, mice and humans behave in an essentially similar way. The study of hybrid enzyme molecules in chimaeric mice has shown that cell fusion is also involved in myogenesis *in vivo* (Mintz and Baker 1967).

Proliferation versus differentiation

When myoblasts differentiate they permanently cease to proliferate. Once a nucleus has entered a myotube it does not normally divide or undergo DNA synthesis again. Under certain conditions, such as after infection with oncogenetic viruses or treatment with mutagens, DNA synthesis can be detected in the nuclei inside myotubes by means of tritiated thymidine labelling and autoradiography, but this 'unscheduled' DNA synthesis is an abnormal event (Yaffe and Gershon 1967; Hahn et al 1971). The withdrawal from the

mitotic cycle which differentiation involves only takes place in myoblasts which are at a certain stage in the mitotic cycle. Microspectrophotometry has shown that the DNA contents of intrasyncytial nuclei in myotubes of chicken origin fall into the diploid range (Strehler et al 1963). This indicates that only cells which are in the G1 period of the mitotic cycle, between mitosis and the beginning of the period of DNA synthesis (Howard & Pelc 1953), become incorporated into myotubes. Autoradiographic examination of chicken myogenic cultures labelled with tritiated thymidine during the process of differentiation has shown that there is a minimum lag period of 3 hours between the end of the last round of DNA synthesis that a myogenic cell undergoes and the time that either daughter cell undergoes cell fusion (O'Neill & Stockdale 1972).

Specialised characteristics of terminally differentiated myotubes.

The differentiated muscle cell is specialised for muscular contraction. In myogenic cultures certain features of the terminally differentiated state are sometimes found in mononucleate cells (Okazaki and Holtzer 1965; Fambrough and Rash 1971), but most of the characteristic properties of muscle appear in the myotubes after cell fusion has taken place.. The contractile proteins have been shown

by the use of fluorescein-labelled antisera to be entirely or very largely localised in the myotubes (Okazaki and Holtzer 1966). Certain enzymes characteristic of mature muscle have also been found to be localised in myotubes by histochemical techniques. Biochemical methods applied to cultures which have been synchronised with respect to differentiation have shown that myosin synthesis and the activities of muscle-associated enzymes increase greatly when myoblasts begin to fuse into myotubes (Paterson and Strohman 1972; Shainberg et al 1971). The ultrastructure of myotubes formed in culture has been studied with the electron microscope and it has been shown that the myofilaments are assembled in cultured cells in the pattern characteristic of muscle tissue (Fischman 1970). Myotubes can interact with nerve cells in culture. Morphologically recognisable neuromuscular junctions are formed (Shimada et al 1969) and acetylcholine sensitivity, which can be demonstrated in myotubes (Fambrough and Rash 1971), has been observed to become localised in response to contact with neuroblastoma cells (Harris et al 1971).

Myogenic cell lines

From the myoblasts of certain species it is possible to derive indefinitely proliferating myogenic cell lines (Yaffe 1968). The cells divide rapidly when passaged at low density but retain the

capacity to differentiate into myotubes when allowed to become confluent. The presence of many of the normal features of myogenic differentiation has been demonstrated in these myotubes, including the accumulation of contractile proteins, elevated enzyme levels and interactions with nerve cells.

Specific problems in myogenesis.

The cells of a rat myogenic cell line have been used to study myogenesis *in vitro*. Four aspects of the control of differentiation in these cells have been investigated

1. the possible existence of a quantal mitosis in myogenesis
2. the role of cell fusion in myogenesis
3. the control of differentiated functions in myoblast x fibroblast hybrid cells

and 4. the expression of muscle genes in erythrocyte/myoblast heterokaryons.

The background to these four problems and the approaches that have been adopted in their investigation are now described. Details of materials used that were common to the different approaches are then given in Chapter II entitled 'Biological Materials'. The four investigations are presented in Chapters III to VI (shared techniques are cross referenced). Chapter VII deals with the application to myogenic

cells of a technique for identifying interphase nuclei on a species specific basis.

Quantal mitosis in myogenesis

It has been suggested that cells only acquire new capacities to synthesise differentiated products after they have been given an external stimulus of some sort and have undergone a division in the presence of this stimulus (Gurdon & Woodland 1968). The casein producing cells of the mammary gland provide an example of the close coupling of proliferation and differentiation on which this theory is based. The epithelial cells of the mouse mammary gland can be cultured and will undergo differentiation *in vitro* in response to three hormones insulin, hydrocortisone and prolactin. These hormones act in sequence. Insulin stimulates DNA synthesis and mitosis in the cells, hydrocortisone then stimulates an increase in rough endoplasmic reticulum and in the Golgi apparatus and prolactin, in conjunction with insulin, finally evokes the production of casein (Mills and Topper 1970). If the initial step of DNA synthesis is experimentally inhibited, the subsequent maturation stages are also prevented from appearing (Turkington & Topper 1967).

A mitosis at which a cell acquires new properties has been termed a 'quantal mitosis'. It has been proposed that many cell types including the myoblast undergo a quantal mitosis before becoming terminally

differentiated (Holtzer and Sanger 1970). This theory is difficult to test in cultures of newly explanted myogenic cells because the cells are likely to be heterogeneous. A cell which differentiates in culture may or may not have undergone a quantal mitosis before it was explanted. From myogenic cell lines, however, it is possible to obtain fairly homogeneous cultures of proliferating myoblasts and to manipulate the environment of the cells so that they switch from proliferation to differentiation. This thesis contains a description of experiments on whether cells which have been manipulated in this way must undergo a quantal mitosis before they can differentiate.

The role of cell fusion in myogenesis

In normal myogenic differentiation myoblasts fuse into syncytia and then accumulate muscle proteins. It has been shown that cells may be artificially fused into syncytia by the use of Sendai virus (Okada 1962). This technique makes it possible to investigate the relationship between cell fusion and the other processes in myogenic differentiation. Myogenic cell lines are particularly suitable for this type of work because they can provide pure cultures of one cell type. Artificial syncytia have been prepared, by virus-induced cell fusion, from the mononucleate cells of a myogenic cell line. The

subsequent behaviour of the syncytia has then been followed to investigate whether artificial cell fusion can stimulate the accumulation of muscle products characteristic of normal myotube formation.

The control of differentiation in hybrid cells

When different cell lines are co-cultivated some of the cells undergo spontaneous cell fusion and the proportion of fusing cells can be markedly increased by treatment with inactivated Sendai virus (Davidson 1969). The two nuclei of a dikaryon may undergo mitosis together and a clone of hybrid cells emerges, containing chromosomal contributions from each of the parent cells. Various selection systems have been devised by means of which such hybrid cell clones may be obtained free of their parent cell types (Littlefield 1966; Handmaker 1971b; Kusano et al 1971). When a hybrid cell line is obtained, it is possible to investigate whether the differentiated properties of the parent cells continue to be expressed in the hybrid. If one parent cell makes a particular protein characteristic of a differentiated state and the other does not make it, do the contributions from the two parent cells act autonomously in the hybrid, do both parental contributions come to make the protein or do neither make it? Table 1 summarises work that has been done in this field.

TABLE 1

Differentiated characteristic studied	Parent cell	Expression of differentiated character	Parent cell	Expression of differentiated character	Expression in hybrid	Re expression after chromosome loss	Reference
Collagen synthesis	Mouse fibroblast 3T6 clone 7	+++	Mouse fibroblast NCTC 2555	+	++		Green et al 1966
		+++	NCTC 2472 cl.6	+	++		
Hyaluronate production	3T6- 7	++	2555	-	+		
		++	2472 - 6	-	+		
Growth hormone	Rat pituitary cell $CH_1^2C_1$	+	Mouse fibroblast Clone 1D	-	-		Sonnenschein et al 1971
Kidney associated esterase ES2	Mouse renal adenocarcinoma RAG	+	Mouse fibroblast LM(TK$^-$)	-	-		Klebe et al 1970b
			Human fibroblast WI 38	-	-	+	
Multiple developmental potentialities	Mouse testicular teratocarcinoma 402 AIII	+	Mouse fibroblast Clone 1D	-	-		Finch and Ephrussi 1967
Multiple developmental potentialities	Mouse testicular teratocarcinoma SIKR	+	Mouse fibroblast Clone 1D	-	-		Jami et al 1973
Malignancy	Mouse tumour cells mammarycarcinoma) sarcoma) lymphoma)	++++	Mouse fibroblast A9	+	+	++++	Harris 1971

TABLE 1 cont (a)

Differentiated characteristic studied	Parent cell	Expression of differentiated character	Parent cell	Expression of differentiated character	Expression in hybrid	Re expression after chromosome loss	Reference
Melanin production	Mouse melanoma B16	+	Mouse fibroblast A9	-	-		Silagi 1967
Melanin production	Syrian hamster melanoma RPMI 3460 - 3	+	Mouse fibroblast Clone 1D	-	-		Davidson et al 1968
			B82	-	-		
			NCTC2555	-	-		
DOPA oxidase		+	Clone 1D	-	-		
Melanin production	2S clone of RPMI3460 - 3	+	Clone 1D	-	±		Davidson 1972 Fougère et al 19
S-100	Rat glial cells		Mouse fibroblasts				Davidson & Benda 1970
	RG6A	+	Clone 1D	-	-		
Glycerol-3-phosphate dehydrogenase							
Base line	RG6A	+++	3T3 - 4(E)	+	+		
			Clone 1D	+	++		
Hydrocortisone inducibility		++	3T3 - 4(E)	-	-		
			Clone 1D	-	-		
Base line	2S clone of RG6A	+++	Clone 1D	+	++		
Hydrocortisone inducibility		++	Clone 1D	-	+		
Immunoglobulin synthesis γG	Mouse myeloma MPC 11	+	Mouse fibroblast 3T3 clone 1T	-	-		Coffino et al 1971

TABLE 1 cont (b)

Differentiated characteristic studied	Parent cell	Expression of differentiated character	Parent cell	Expression of differentiated character	Expression in hybrid	Re expression after chromosome loss	Reference
Immunoglobulin secretion.							
A type heavy chain	Mouse myeloma	+	Mouse fibroblast	+	-		Periman 1970
λ light chain	MOPC 315		Clone 1D				
anti-DNP antibody secretion		++		-	+	?	
Immunoglobulin secretion γG	Mouse myeloma RPC-5 clone 4	+	Mouse lymphoma EL4	-	±		Mohit & Fan 1971 Mohit 1971
free K chains		+		-	+		
Tyrosine aminotransferase (histochemically detected)	Rat hepatoma HTC	+	Rat liver epithelial cell BRL - 62	-	- (heterokaryons examined before nuclear fusion)		Thompson & Gelehrter 1971
Tyrosine aminotransferase	Rat hepatoma HTC		Mouse fibroblast				
high base line	AR - 1	+	3T3 - 4E	-	-		Benedict et al 1972
dexamethasone inducibility		+		-	-		
Aryl hydrocarbon hydroxylase benz[α]anthracene inducibility		-		+	++		

TABLE 1 cont (c)

Differentiated characteristic studied	Parent cell	Expression of differentiated character	Parent cell	Expression of differentiated character	Expression in hybrid	Re expression after chromosome loss	Reference
Arylhydrocarbon hydroxylase; benz [α] anthracene inducibility	Human choriocarcinoma JEG - 3	+	Human fibroblast VA - 2	-	-		Wiebel et al 1972
	Hamster embryo cell OBP	+	Hamster kidney cell BHK	-	-		
	Mouse fibroblast 3T3 - 4C2	+	Mouse fibroblast A9	-	++		
Tyrosine aminotransferase high base line	Rat hepatoma H4IIEC3 Clone Fu5	+	Mouse fibroblast 3T3 - 4E	-	-		Schneider & Weiss 1971
dexamethasone inducibility	Clone Fu5	+		-	-		
Aldolase B	Fu5	+	Mouse fibroblasts 3T3 - 4E	-	-		Bertolotti & Weiss 1971
			Clone 1D	-	-		
			Rat liver epithelial cell BRL - 1	-	-		
Tyrosine aminotransferase high base line	Fu5	+	BRL - 1	-	-		Weiss & Chaplain 1971
dexamethasone inducibility		+		-	-	+	

TABLE 1 (d) cont

Differentiated characteristic studied	Parent cell	Expression of differentiated character	Parent cell	Expression of differentiated character	Expression in hybrid	Re expression after chromosome loss	Reference
Albumin production	Fu5	+	3T3 - 4B	-	+		Peterson & Weiss 1972
	2S clone of Fu5 Fu5 - 5 cl.1B	+	3T3 - 4B	-	- +		
Electrical excitability of cell membrane	Mouse neuroblastoma N4TG 1	+	Mouse fibroblast B82	-	+		Minna et al 1971; 1972
Acetyl cholinesterase		+		-	+		
Neurite formation		+		-	+		

It has generally been found that differentiated activities are extinguished when the cells that express them are hybridised with cells not differentiated in the same way. The most thoroughly investigated example of this finding is the case of the kidney-associated enzyme Es2, which is produced by a mouse renal adenocarcinoma cell line, RAG, but is absent from mouse L cell fibroblasts and from the human lung fibroblast cell line, WI38. Hybrids between RAG and either of the two fibroblast cell lines do not show Es2 activity, so it appears that the two cell types are able to interact and that the fibroblast's failure to express the enzyme is dominant over the adenocarcinoma cell's capacity to do so (Klebe et al 1970b). The simplest interpretation of this result is that the fibroblast produces suppressing conditions which at some level prevent Es2 production in this cell type and that these conditions also suppress the production of Es2 by the adenocarcinoma contribution in the hybrid cells.

It might be argued that fusion in some way damages the cells and that Es2 is not produced in the hybrids because of some non-specific reason that has nothing to do with the way that Es2 production is controlled in the fibroblast. This does not appear to be the case. Fusion per se does not abolish differentiated functions, for if two cells differentiated in the same way are fused together, the 2S cell

that is produced and its progeny remain differentiated (Peterson and Weiss 1972). The results of further work done on the RAG X WI38 hybrids are even more convincing on this point. The human chromosomes are progressively lost from the human x mouse hybrid cells (Weiss and Green 1967) and when they are lost from the RAG X WI38 hybrids the Es2 activity reappears. Reappearance of Es2 has been correlated with the loss of the human chromosome C10. This shows that the fusion of the two cells has not in some way irreparably damaged the RAG cell. It also shows that the controlling factors suppressing Es2 activity in the hybrid are associated with a particular chromosome. It does not prove that the factors associated with the C10 chromosome are the only ones limiting Es2 activity in the WI38 cell. If this were the case the loss of the C10 chromosome might be expected to allow the expression of human Es2. This aspect of the system has not been unravelled.

The fact that the RAG contribution to the hybrid cell can recover its ability to make Es2 when the human chromosomes are lost shows that its own hereditary epigenetic programming can be maintained in the hybrid cell even when its expression is suppressed. At this level the mouse cell has behaved autonomously in the hybrid since its own capacity to repress Es2 activity, which would be expressed in a mouse fibroblast for instance, has not been evoked by contact

with the contents of the human fibroblast.

Much of the work that has been done on the expression of differentiated functions in hybrid cells has involved the use of fibroblast lines as the parent cells not expressing the function under study. This fact raises the question of the generality of the findings. Work with immunoglobulin producing cells indicates that if a differentiated function is extinguished when a cell that expresses it is fused with a fibroblast, this does not mean that it would be extinguished in hybrids with all other cell types. Myeloma cells secreting γG have been hybridised with fibroblasts and the hybrids were found not to synthesise detectable immunoglobulin (Coffino et al 1971). Another line of myeloma cells which secretes γG and free κ chains was fused with a lymphoma line which did not secrete any immunoglobulin. In this case the hybrid cells were found to secrete κ chains and two hybrid subclones secreted complete γG molecules (Mohit 1971). These two results raise the possibility that the mechanism by which a differentiated function, such as immunoglobulin synthesis, is suppressed in two different cell types such as the fibroblast and the lymphoma may be completely different. This type of difference may represent an important aspect of epigenetic control <u>in vivo</u>. In the interpretation of such findings there is, however, a need for caution, which stems from the capacity of some cell lines to

change in culture. Some lymphoma cells produce immunoglobulins (Hinuma & Grace 1967), and cell lines which produce immunoglobulins are known sometimes to lose this capacity in culture (Coffino and Scharff 1971). It is conceivable that the lymphoma used by Mohit synthesised immunoglobulin at one time and has lost the ability to do so for some trivial reason. If this were the case, the epigenetic control mechanisms which prevent the expression of the immunoglobulin genes in other cell types, such as the fibroblast, would not be expected to be present in the lymphoma.

Even when the parental cells differ sharply in the expression of differentiated characteristics, certain differentiated functions have been shown to persist in hybrid cells. Fu-5 hepatoma cells express four differentiated functions which are characteristic of liver and which are not found in fibroblasts, a high base line of tyrosine aminotransferase activity, 4-6 fold inducibility of tyrosine aminotransferase by the hormone dexamethasone, a liver specific aldolase and albumen synthesis. The first three of these are suppressed in hepatoma x fibroblast hybrids (Schneider & Weiss 1971; Bertolotti and Weiss 1971), but in the same hybrid cells albumen synthesis persists (Peterson & Weiss 1972). This shows that the expression of at least one liver specific function is not extinguished in hepatoma x fibroblast hybrids and hence also that all the differentiated character-

istics are not controlled by the same mechanism in the hybrid cell. The most striking example known of the retention of differentiated properties in hybrid cells occurred in neuroblastoma x fibroblast hybrids. Neuroblastoma cells were fused with mouse L cell fibroblasts and the hybrid cells were found to retain the electrical behaviour of the cell membrane of the neuroblastoma parent, to show neurite formation and to contain cytochemically demonstrable acetylcholinesterase (Minna et al 1972).

The myoblast provides an interesting subject for cell hybridisation because of the many characteristic differentiated features which it displays in culture, such as cell fusion, elevated enzyme levels and the accummulation and assembly of contractile proteins. The capacity of hybrid cells to express aspects of myogenic differentiation was studied, using hybrids made between mouse fibroblasts and the cells of a rat myogenic cell line. Two different L cell sublines, A9 and B82, were used as the fibroblast parents. B82 was the line used in the experiments that showed the persistence of neurological differentiation in neuroblastoma x fibroblast hybrids (Minna et al 1972).

The expression of muscle genes in erythrocyte/muscle cell heterokaryons

The nuclei of avian erythrocytes can be experimentally introduced into mammalian cells such as the human HeLa cell by means of haemolytic concentrations of inactivated Sendai virus. Most of the erythrocyte cytoplasm is lost and the nucleus is effectively brought into the HeLa cell when the erythrocyte ghost is invaded by the HeLa cell's cytoplasm (Schneeberger & Harris 1966). Normally the erythrocyte nucleus is small, contains dense chromatin, does not synthesise DNA and is not usually considered to synthesise RNA, although a recent report challenges this belief (Madgwick et al 1972). When it is introduced into the cytoplasm of another cell, however, it swells, its chromatin becomes dispersed and nucleic acid syntheses may be resumed (Harris 1967).

Protein synthesis directed by the avian genome has been shown to take place in heterokaryons constructed from chicken erythrocyte nuclei and mammalian cells. The question therefore arises as to whether an erythrocyte nucleus placed into another cell type will make differentiated products characteristic of the erythroblast from whence it originally came, whether it will make proteins characteristic of the cell type into which it has been placed, or whether it will only make the proteins that are common to

both cell types. It has been shown that haemoglobin synthesis is suppressed in heterokaryons formed from chicken erythrocyte nuclei and mouse tissue culture cells (Harris 1970b). The chicken specific products that have so far been detected after the reactivation of chicken erythrocyte nuclei in other cells, inosinic acid pyrophosphorylase, surface antigens (Harris 1970b) and interferon (Guggenheim et al 1968) are all characteristic of a wide range of cell types (De Maeyer 1967) and could well have been made by the immature erythroblast as well as the host cell type. It remains to be proven whether the cytoplasmic environment provided by a differentiated cell can reprogram an erythrocyte nucleus to entirely new differentiated syntheses. Paramecium provides an example of a related phenomenon where this sort of reprogramming does occur: when a nucleus from a cell expressing one surface antigen enters a cell expressing another (at conjugation), the allele to the 'host's' active gene in the introduced nucleus becomes activated (Beale 1954).

Myogenesis is a particularly suitable system for examining this question since the proteins characteristic of the differentiated state are accumulated by post-mitotic cells. There is evidence that some of the (undifferentiated) synthetic activities of erythrocyte nuclei take several days to appear in

heterokaryons (Harris 1970b), although this does not apply to interferon which is found 24 hours after the erythrocyte nuclei have been introduced (Guggenheim et al 1968). When erythrocyte nuclei are introduced into proliferating cells they synthesise DNA but mostly undergo 'premature condensation' at the next mitosis and make little or no contribution to the daughter cells (Schwartz et al 1971). In the work on the appearance of chicken cell surface antigens and inosinic acid pyrophosphorylase, the host L cells were therefore X-irradiated so that mitosis was prevented. The interpretation of cellular activities, or in particular the absence of activities, presents additional problems if the cells involved have been previously irradiated. This difficulty does not apply to the post-mitotic myotube. Another advantage of the myogenic system for the study of this problem is that the differentiated cell is a syncytium so that the ratios according to which the host and introduced nuclei are combined can be varied over a considerable range. Work on hybrid cells has shown that the expression of differentiated functions in such cells can depend on the dosages according to which the two parent cell types are combined (Peterson and Weiss 1972; Davidson 1972). If a small number of erythrocyte nuclei can be combined with a larger number of myogenic nuclei in a myotube then this may

favour the expression of the muscle genes in the erythrocyte nuclei.

Chicken erythrocyte nuclei have been introduced into the myoblasts and myotubes of a rat cell line and the cells have subsequently been examined for the appearance of chicken muscle specific antigens. These experiments are described.

CHAPTER II

Biological Materials

Maintenance of myogenic cell line

Details of cell culture techniques

The myogenic cell line, L_5, derived by Yaffe from a newborn rat, was used for the experiments described in this thesis. A variety of culture conditions were used until the requirements for proliferation and differentiation of this cell line had been established. The conditions found to be satisfactory were as follows:

Medium:

Eagle's minimum essential medium, Hanks based and with non-essential amino acids added, was bought in 10X concentrated form from Wellcome Laboratories and diluted in autoclaved twice-distilled water. (See page 142 for medium recipe)

Buffer system:

Sterile sodium bicarbonate solution was added to the medium to make a final concentration of 1.1 gm per litre. The incubator was gassed with a mixture of approximately 8% CO_2 in air. Air was supplied from a Hyflo pump and passed through a flow meter. CO_2 from a cylinder was passed through a pressure control valve and flow meter and then mixed with the air. The mixture was bubbled through a wash bottle containing a solution of sodium bicarbonate

and phenol red and then passed into the incubator. The wash bottle provided a useful means for checking that gas flowed correctly and that CO_2 was being mixed in at the correct ratio.

Temperature:

A water jacket insulated incubator (Laboratory Thermal Equipment) was used. A tray filled with a dilute solution of copper sulphate was placed at the bottom to maintain a water saturated atmosphere. The temperature was kept at 36.5°C.

Dishes:

Primary cultures of myogenic cells differentiate most effectively if the substratum is coated with collagen (Hauschka & Konigsberg 1966) or gelatine (Richler & Yaffe 1970). The L_5 myogenic cell line was found to grow and differentiate on dishes of two different brands, Nunclon & Sterilin, even if they had not been coated. The cells behaved differently on different brands of dish; for instance, they adhered far less strongly to Falcon dishes than to the Sterilin ones which have been routinely used.

Antibiotics:

The infection of cell lines with Mycoplasma is a serious problem. It was found to occur with L_5 cells and appeared to cause them to stop differentiating. Kanamycin is reported to inhibit the growth of these organisms (Paul 1970) and this

antibiotic (Flow labs.) was routinely added to the medium at a final concentration of 10 μg per ml. Although it can be argued that routine incorporation of antibiotics invites the development of resistant strains of microorganisms, it does provide protection from occasional accidental infection, which might take some time to become apparent. Infections of fungi and bacteria usually manifest themselves within 24 hours and so cultures containing them can be discarded. Accordingly no antibiotics were used specifically against these agents, particularly because penicillin actually promotes the development of L forms (Paul 1970).

Serum:

Foetal bovine serum was obtained from Biocult. Batches of the serum vary considerably in their ability to support the growth of L_5 cells. It was found that when healthy differentiating cultures were transferred to medium containing an unsuitable serum, the cells continued to differentiate and the deficiencies of the serum took some time to show up. To test a batch of serum adequately it was necessary to grow up clones in it from the single cell stage to the stage of differentiation. It was therefore important for the supplier to hold large stocks while batches were being tested.

Routine culture:

Fresh medium was normally given to cells on

every third day. Confluent cultures were subcultured as follows: the dishes were rinsed with Dulbecco A solution (Oxoid) and the cells were then detached by treatment with a 0.25% solution of trypsin (Difco 1:250) in Dulbecco A. As soon as the cells on a dish had become dispersed, an appropriate volume of fresh medium was added and the suspension of cells was transferred to new dishes. If the volume of trypsin solution used was sufficiently small, it was found to be unnecessary to remove the trypsin before suspending the cells in fresh medium.

Storage:

Stocks of cells were stored at -65°C. Cell samples were suspended in ordinary culture medium containing 10% serum and 10% dimethyl sulphoxide or glycerol and placed into 1 ml glass ampoules which were sealed and frozen. In order that the rate of cooling should be low, the ampoules were wrapped with insulating material for the first few hours in the freezer. To recover a sample of cells, an ampoule was warmed rapidly to room temperature and the contents were plated out in ordinary medium.

Cloning:

In the maintenance of L_5 cells, it was frequently found to be necessary to isolate vigorous myogenic cells from a heterogeneous population. This was achieved by isolating clones of cells with the required characteristics. Cells were thoroughly

dispersed in trypsin solution, counted in a haemocytometer, diluted appropriately and plated on new dishes. For routine cloning it was sufficient simply to disperse a half confluent dish of cells - confluent cultures will not disperse evenly - and to replate them at from 10^{-3} to 10^{-4}. The new sparse cultures were then fed with fresh medium on every third day and when the single cells had multiplied to form small patches and undergone differentiation, suitable clones for further propagation were selected. The medium was then replaced with trypsin solution and after a few minutes - the exact time depending on the temperature of the trypsin - each selected clone was scraped gently off the dish into a sterile finely drawn pasteur pipette and transferred to a new dish. At first, feeder layers of 25.10^3 Xrayed (6000r) fibroblasts per 6 cm dish were used, but these were later found to be unnecessary and the cells appeared to have become adapted to grow in the tissue culture environment even when plated alone. Cloning efficiencies of up to 100% were obtained.

Effects of serum concentration

Cells of the L_5 myogenic cell line proliferated rapidly in medium containing 10% of foetal bovine serum of a suitable batch. When frequently subcultured their generation interval appeared to be approximately one day. When cultures were allowed

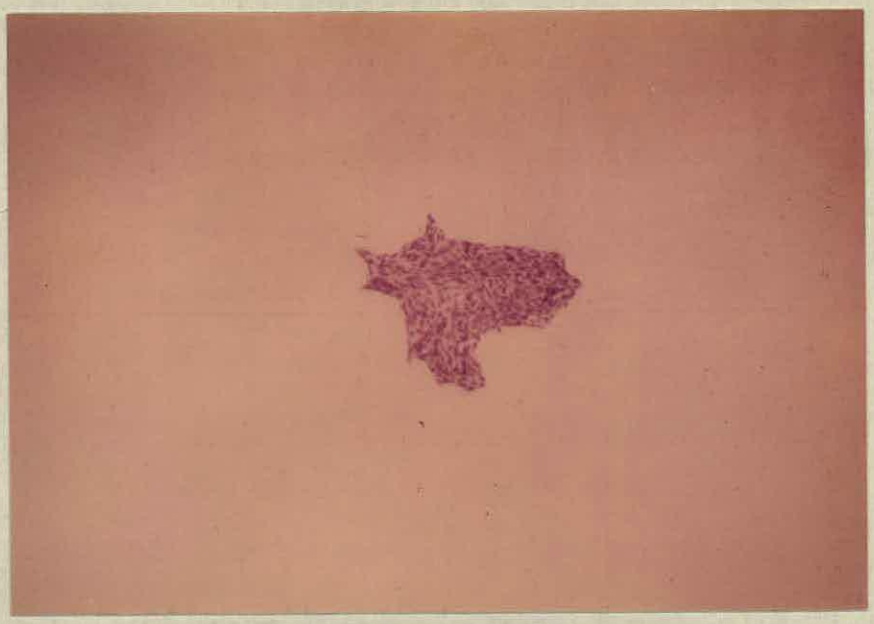

Plate 1. (Zeiss stereomicroscope IV) ×33

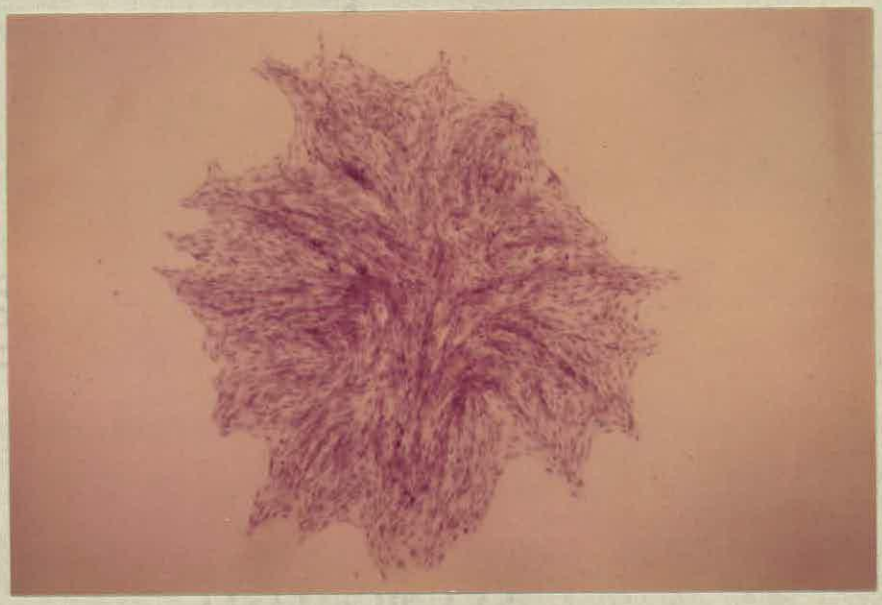

Plate 2. ×33

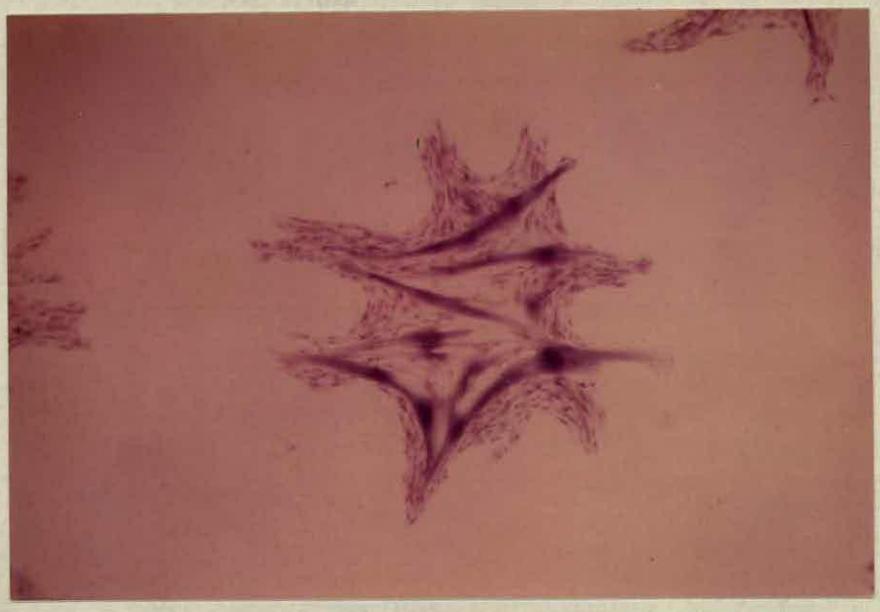

Plate 3. ×33

to become dense in 10% serum, the cells differentiated. It was found,however, that differentiation was more rapid and extensive if the serum concentration was reduced. The difference which the concentration of serum made to the behaviour of the cells was most clearly seen when dishes were sparsely covered with patches of cells. This was because other factors connected with the distribution of the cells are also involved in determining whether the cells differentiate or proliferate. First of all the myoblasts proliferate most rapidly if the number of cells per dish is kept fairly low but secondly the cells need to be packed next to each other if they are to make the cell to cell contacts which cell fusion involves. Dishes containing sparsely distributed patches of densely packed cells represented a compromise to minimise the influence of these two effects and hence to show the influence of serum concentration more clearly. Dishes of this sort were obtained when clones of cells were grown. Plate 1 shows a clone of L_5 cells grown in 10% serum for 6 days and then fixed and stained. Plates 2 and 3 show similar clones cultured in parallel for the first 6 days, after which the clone on plate 2 was cultured for a further 4 days in 10% serum, while that on plate 3 was transferred for 4 days to 2% serum. The higher concentration of serum appears to have stimulated the cells to continue to proliferate while the lower concentration has

Plate 4. × 10

Plate 5. × 10

allowed them to express their capacity to differentiate. A similar effect of serum has been found for neuroblastoma cells. Serum is essential for the proliferation of these cells in culture but if it is removed from the medium the cells express their differentiated functions of neurite formation and acetyl cholinesterase activity much more strongly (Seeds et al 1970; Blume et al 1970).

Cell interactions in culture

Experiments on the effects of serum concentration on clones of L_5 cells produced evidence that there can be cellular interactions between clones as well as within them. Konigsberg (1971) has found that patches of cultured quail myoblasts produce a diffusible substance which stimulates themselves or patches of other similar myoblasts to differentiate. Differentiation of the myoblasts of the L_5 rat cell line may also involve such a substance. The evidence that this may be the case is that clones of similar cells, cultured in parallel in the same concentration of serum, behave differently according to the number of the clones on the dish. Plates 4 and 5 show clones of cells originally plated from the same suspension of cells and then both cultured for a total of 10 days in 10% serum. It can be seen that proliferation was more extensive in the clones of the sparser culture and that differentiation has not been completely prevented by the 10% serum in those of the denser one. This

type of experiment does not show whether the clones interact by depleting a mitogenic factor in the medium or by secreting a factor which stimulates differentiation. In view, however, of Konigsberg's demonstration that myogenesis promoting substances can be secreted into the medium, the latter interpretation seems the more likely one.

Primary rat myogenic cultures and attempts to establish myogenic cell lines

The reason why some cells overcome the ageing block is unknown. The process has been observed to occur more frequently in the cells of some species than in those of others (Green & Todaro 1967). Rodent and carnivore cells are supposed to make cell lines fairly frequently, human cells very rarely, and bird cells never do so. The process is a rare event, even for species which make cell lines relatively frequently, and so in order to obtain a cell line it is important to have a large number of cells in culture at the time when they are dividing slowly and when the immortal nature of an emerging line will therefore show up. This is particularly difficult to manage for the myogenic cell type because of the extremely sparse conditions under which newly explanted myoblasts have to be kept. If they become dense, they differentiate.

A number of attempts to establish myogenic cell lines were made in the following way. Primary

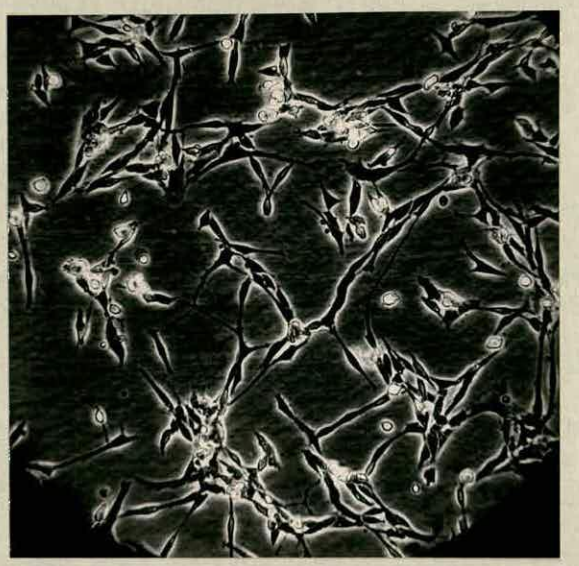

Plate 6. Cells which took more than 40 minutes to attach to the dish.

Wild M40 phase contrast inverted microscope magnification x160

Plate 7. Cells which attached to the dish within 40 minutes. x160

cultures were prepared from newborn rats. Whole thighs and arms from freshly killed animals were removed and carefully skinned under sterile conditions. They were then placed in trypsin solution and stirred vigorously at room temperature. At half hour intervals the trypsin was renewed and the old trypsin solution, with the cells that had become dispersed in it, was centrifuged (200 g for 5 mins). The precipitated cells were resuspended in medium and put onto collagen coated dishes. Between one and three days later the primary cultures were fractionated to purify the myogenic cell type. The cells were trypsinised off the dishes, resuspended in medium and put back onto another dish. This dish was left undisturbed in the incubator for 40 minutes. The medium was then swirled round, removed and put onto a further collagen coated dish. Plates 6 and 7 show the difference, 24 hours after the fractionation procedure, between the cells which attached to the dish in 40 minutes and those that took longer to do so. Most of the cells in a primary culture are myoblasts and fibroblasts and whereas many of the fibroblasts attach in 40 minutes far fewer of the myoblasts do so. Attention was now focussed on the dishes with the enriched myoblast population. The cells were allowed to proliferate, but as soon as they became dense enough to start differentiating they were subcultured. This was repeated whenever myotube formation began. It was

found to be difficult to prevent myoblasts which had been in culture for a long time from differentiating. Even when the cells were plated under very sparse conditions, on feeder layers of X-rayed fibroblasts, differentiation began within a few days. This meant that it was necessary to trypsinise and replate the cells very often if they were not all to be lost as myotubes. On the other hand, unlike cells which have become adapted to tissue culture conditions, quite a large proportion of these myoblasts did not survive subculture and so were lost in that way. In one way or the other all the cells eventually died out. The L_5 rat myogenic cell line derived by Yaffe (1968) using a similar procedure was growing satisfactorily and so attempts to isolate new lines were suspended. Yaffe treated the precursor cells of some of his first myogenic cell lines, including L_5, with methylcholanthrene. However, he has since established other lines without the use of this compound and its importance in the early work is not clear.

Differences between myogenic cell lines and newly explanted myogenic cells

Myogenic cell lines are not only different from newly explanted myoblasts in their capacity for indefinite proliferation. They are also more adhesive, they can be cloned with a much higher efficiency and can be grown at very low densities without feeder

layers. Most workers use embryo extract for the culture of myogenic cells (de la Haba & Amundsen 1972) but this has been found to be unnecessary for the L_5 cell line. Other differences that myogenic cell lines can acquire are changes in karyotype (Richler and Yaffe 1970) and the capacity to form tumours when injected into animals (G.Kessler - personal communication). All these alterations in the cells appear to be acquired during their long period in culture. The question arises as to whether the investigation of the biology of cell lines can give information which applies to ordinary myogenic cells. Cells of the L_5 myogenic line differentiate more slowly than newly explanted myogenic cells. They do however show the general characteristics of myogenic differentiation in vitro. The mononucleate myoblasts proliferate but retain the capacity to differentiate. They can withdraw from the mitotic cycle, undergo cell fusion, accumulate contractile proteins and attain elevated levels of muscle enzymes. It seems likely that the ways in which the inheritance and expression of these activities are controlled in the L_5 cells are similar to the ways in which they were controlled in the newly explanted rat myoblasts from which they were originally derived, but the possibility that this is not the case cannot be completely dismissed.

Failed myoblasts

When clones of cells were isolated from mass cultures of L_5 cells some of the clones did not show myotube formation on the dish on which they were grown up from the one cell stage. These clones did not seem to be epigenetically distinct, however, because when transplanted to separate dishes they grew and differentiated in the ordinary way. Similar results have been reported for other rat myogenic cell lines (Richler and Yaffe 1970). The reasons why patches of myoblasts of equal size, on the same dish and sharing the same medium, do not all behave in the same way are not clear.

Apart from the local variability that can arise in the expression of the myogenic potentiality of L_5 cells, the cells were also sometimes found to vary in their potentialities. Some of the undifferentiated clones turned out to be clones of genuinely 'failed myoblasts' which would not differentiate under any of the conditions which induced myotube formation by normal L_5cells. A number of such clones have been isolated and maintained over many weeks without showing any visible sign of differentiation. Since non-myogenic cell lines of mouse origin were being cultured in the same incubator as the L_5 cells, it appeared possible that the L_5 cultures had become contaminated with mouse cells. Examination of the glucose phosphate isomerase phenotype (see page 82)

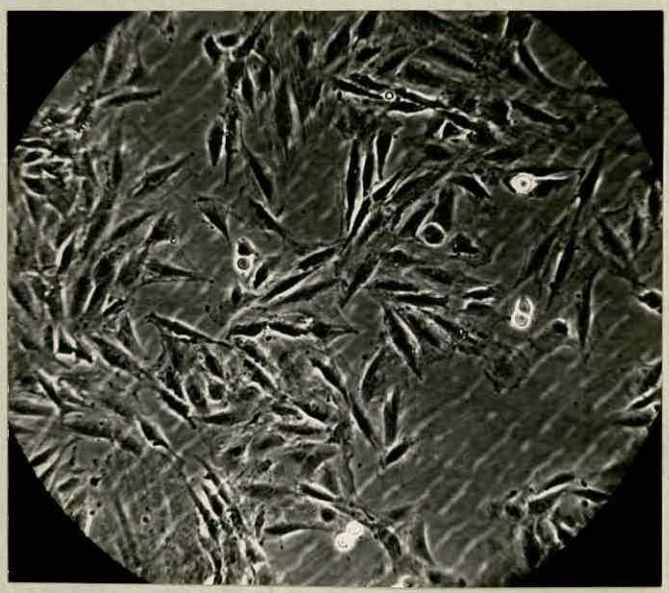

Plate 8. L5.1.1.5f1 ×160

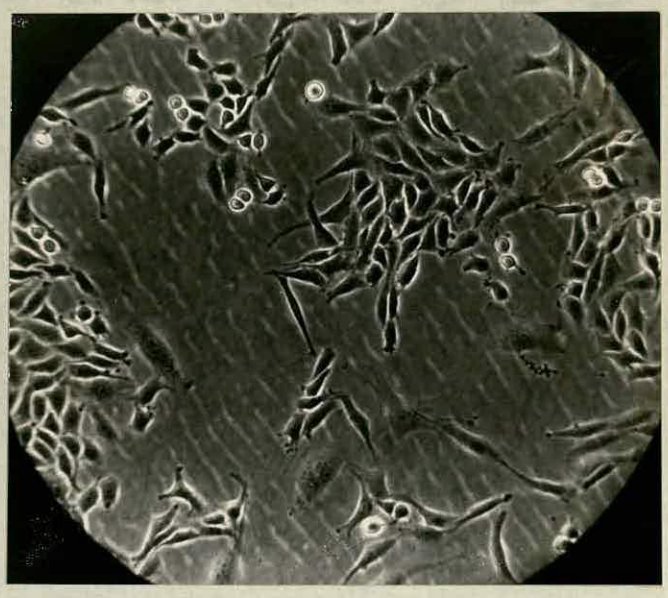

Plate 9. L5.1.1.5.5.2.1.9f1 ×160

of the non-myogenic clones showed that they were of rat origin. Different clones of failed myoblasts have different properties. Plates 8 and 9 show two clones of failed L_5 cells designated $L_{5.1.1.5.}{}^{f}{}_{1}$ and $L_{5.1.1.5.5.2.1.9}{}^{f}{}_{1}$ respectively. The latter clone proliferates more rapidly than the former, and the cells have a more refractile, less flattened appearance in culture.

A number of attempts were made to induce failed myoblasts to differentiate. When cultures were grown to confluence and treated with 2% serum for many days, the cells failed to initiate differentiation. The cells were also plated on one half of a dish, with ordinary L_5 myoblasts on the other half. When the two colonies had grown until they had come into contact with each other myotube formation was restricted to one side of the dish and the line along which the differentiating cells met the non-differentiating ones could clearly be seen under the microscope. Finally, an attempt to incorporate failed myoblasts into the myotubes formed by newly explanted cells was made. L_5 cells from a differentiating clone and also from a failed clone were grown in medium containing tritiated thymidine and then mixed into actively differentiating primary cultures of rat myoblasts. After a period of differentiation, the cultures were fixed and processed for autoradiography (see page 48). When ordinary L_5 myoblasts had

Plate 10. × 500

been mixed into the culture, labelled nuclei were found within the myotubes (see plate 10) but when failed myoblasts had been used, none of the labelled cells were found to have been incorporated.

The reasons why failed myoblasts arise from myogenic cell lines are not understood. It seemed possible that infection by Mycoplasma could be the cause. However, two extended examinations of $L_{5.1.1.5^{f}1}$ by Dr Stewart of the Virology Department at the Edinburgh City Hospital failed to show any evidence of Mycoplasma contamination. Cell lines which are differentiated in other ways also 'fail' and the frequency with which they do so varies from line to line (Moore 1964; Coffino & Scharff 1971). The fact that undifferentiated clones of myoblasts on a dish are not necessarily failed makes it difficult to screen large numbers of clones, but the rate of failure of L_5 cells did appear to vary. Although failed myoblasts arose during normal culture, certain treatments such as sending the cells from one laboratory to another or freezing them for storage always produced a high proportion of failed cells. To ensure the maintenance of a high proportion of active myogenic cells in mass cultures, actively differentiating subclones were continuously reisolated.

Difficulties of culturing myogenic cell lines

The maintenance of the L_5 myogenic cell line presented serious difficulties. The cells sometimes ceased to differentiate or only differentiated sporadically. Under these circumstances it was sometimes possible to isolate satisfactory myogenic subclones but this was not always the case. When the subclones also only showed sporadic differentiation, it was necessary to return to stocks of cells frozen down before the problems arose. However, freshly thawed cells had also to be cloned if highly myogenic mass cultures were required. Since it took several weeks to propagate mass cultures from a new clone, the isolation procedures used to recover active myogenic cultures were extremely time consuming. These difficulties are not restricted to this laboratory (Yaffe - personal communication) and they are the main stumbling block in any work with myogenic cell lines. The experiments described in this thesis were performed during periods when the cells were differentiating satisfactorily but many wasted experiments were also performed when the cells ceased to differentiate in the middle of the work.

Culture of Sendai virus

Sendai virus, obtained from Dr Baton of the Royal Dick Veterinary College, was propagated in the allantoic cavities of chicken embryos. Batches were

prepared as follows: allantoic fluid from the previous culture was diluted in sterile Dulbecco A solution to from 10^{-2} to 10^{-3} and filtered through a sterile Millipore filter (pore size 0.22μ) in a Swinney filter holder. 0.1 ml of the filtered suspension was injected into the allantoic cavity of each of 48 candled fertile eggs that had been incubated at 38°C for 9 days. The holes in the egg shells were sealed with molten wax and the eggs were incubated for a further three days at 35.5°C and then chilled at 4°C overnight. The allantoic fluid was pooled and centrifuged at 500 g for 10 minutes to remove large particles and blood cells from suspension. The precipitates were discarded. The fluid (about 300 ml) was recentrifuged at 35,000 g for 35 minutes and the supernatants were discarded. The pellets were suspended in about 6 ml of sterile Hanks solution made up without glucose (Harris et al 1966) but with the addition of 0.5% bovine serum albumin (Neff and Enders 1968). The final suspension was stored in 0.3 ml lots at -65°C.

Virus titration was performed by haemagglutination in Linbro plastic haemagglutination trays. Virus suspension was thawed and serially diluted in Dulbecco A+B solution by the doubling dilution technique so that a series of half filled 1 ml wells were obtained, each containing 0.5 ml of virus suspension at half the concentration to that in the

previous well. 0.5 ml of a 0.5% suspension (v/v) of erythrocytes collected from newly hatched chicks was then added to each well and the whole haemagglutination tray was placed at 4°C for 6 hours. One HAU was defined as the minimum amount of virus which agglutinates the erythrocytes when placed in a total volume of 1 ml of Dulbecco A+B solution in a well of a Linbro haemagglutination tray together with newly hatched chickens' erythrocytes at a final concentration of 0.25%. Virus suspensions prepared as described above usually gave a titre of $8\text{-}16.10^4$ HAU per ml.

HAU titre is not directly correlated with the cell fusing factor in Sendai virus. It has been reported that even when the virus is stored at -65°C, the fusion factor decays significantly (Neff & Enders 1968). No critical experiments were done on this question but variable results were at first obtained in experiments on virus-induced cell fusion, especially where the dose of virus was important. A new batch of virus was therefore prepared at least once a month and this appeared to make experiments more accurately repeatable.

Virus was inactivated by ultraviolet irradiation just prior to use. A bottle of 0.3 ml of suspension was thawed and pipetted onto a sterile tissue culture dish to make a thin bubble-free layer. This was then irradiated with a Camag u.v. lamp (λ = 254 mμ)

at 12 cms for 3 minutes, swirled thoroughly, irradiated for a further 3 minutes and then diluted in saline or medium to the appropriate concentration for use.

Preparation of contractile proteins and antisera to them

Preparation of myosin and actomyosin

Myosin and actomyosin were prepared by the methods of Finck (1965a) with the modification that dithiothreitol (Calbiochem) was added to all the solutions at a concentration of 0.0005M. The method of extraction exploits the solubility of the proteins in solutions of KCl of high concentration and the purification procedure involves repeated differential precipitation at lowered ionic strengths. All the procedures were conducted at 4°C. Precipitated proteins were redissolved after centrifugation with the aid of a Potter Elvejhem glass Teflon homogeniser (Kontesglass, New Jersey).

Musculature from one freshly killed chicken, obtained from Dr Gilbert of the Poultry Research Centre, was chilled in ice, ground once in a stainless steel meat mincer and extracted with 3 times its own volume of an ice cold solution of the following composition: 0.3M KCl, 0.095M KH_2PO_4, 0.075M K_2HPO_4 & 0.001M ethylenediaminetetra-acetic acid (EDTA) (pH 6.5 ionic strength 0.57).

Extraction was stopped after 10 minutes by the addition of 1 volume of water (containing 0.0005M dithiothreitol). The muscle debris was removed by centrifugation and used for preparing actomysin. The supernatant was used for preparing myosin.

Myosin

After the initial extraction the crude myosin was precipitated by dilution of the solution with 12 volumes of cold water (containing dithiothreitol) and allowed to settle overnight. The precipitated protein was collected by centrifugation and re-dissolved in a solution of 0.81M KCl, 0.11M KH_2PO_4, 0.1M K_2HOP_4 & 0.001M EDTA (pH6.5 ionic strength 1.15). The solution was clarified by centrifucation (20,000g 1 hour). Actomysin was then precipitated from the solution by dilution to ionic strength 0.28 and removed by centrifugation. Myosin was precipitated by further dilution to ionic strength 0.04. It was collected by centrifugation, redissolved at ionic strength 1.15 and cycled two more times through the fractional precipitation procedure at ionic strengths 0.28 and 0.04. It was then dialysed against a solution of 0.5M KCl and 0.02M Tris(hydroxymethyl) aminomethane-HCl (Tris) pH7.3, clarified by centrifucation and fractionated with ammonium sulphate by the method of Nauss et al (1968). A saturated solution of $(NH_4)_2SO_4$ containing 0.001M EDTA pH7.0 was added slowly. The fraction precipitating

between 38 and 50 saturation was collected by centrifugation, redissolved in 0.5M KCl, 0.02M Tris pH7.3 and dialysed against more of this solution to remove traces of $(NH_4)_2SO_4$. This preparation was used as chicken myosin for immunisation and electrophoretic analysis on polyacrylamide gels.

Actomyosin

The muscle debris remaining after the initial extraction of crude myosin was further extracted for 20 hours with twice its own volume of a solution containing 0.3M KCl, 0.17M K_2HPO_4 & 0.001M EDTA (pH 8.6 ionic strength 0.8). The solution was centrifuged (1,000g 20 minutes) and the precipitate was discarded. The supernatant was diluted with water to an ionic strength of 0.25 and the precipiated actomyosin was collected by centrifugation. The protein was redissolved at ionic strength 0.8, the solution was clarified by centrifugation (20,000g 1 hour) and the actomyosin was reprecipitated at ionic strength 0.13. It was then redissolved, reprecipitated and the final solution was dialysed against a solution of 0.5M KCl, 0.02M Tris pH7.3. The solution was fractionated with ammonium sulphate. The fraction salting out at 38% saturation was redissolved and dialysed to remove traces of $(NH_4)_2SO_4$. This preparation was used as chicken actomyosin for immunisation and electrophoretic analysis on polyacrylamide gels.

Storage of protein preparations

Myosin and actomyosin solutions were stored at -20°C after dialysis against 0.5M KCl,0.02M Tris pH7.3 containing 50% glycerol. Protein concentrations were determined before dialysis from the absorbancy of the solutions at 280mμ. The standards used were $E^{1\%}_{1cm}$ = 5.60 for myosin (Small et al 1961) and $E^{1\%}_{1cm}$ = 17.1 for actomyosin (H.A.John, unpublished results).

Electrophoretic analysis of myosin and actomyosin preparations

The chicken myosin and actomyosin preparations were analysed by Dr H.A.John using the technique of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Weber and Osborn 1969). The results are shown on plate 11. The myosin showed an electrophoretic pattern similar to those published for chicken myosin (Dow & Stracher 1971). The prominent band which migrated only a short distance represents the large subunit of myosin and the three rapidly migrating bands represent the three light chains of myosin. The actomyosin preparation contained two components with intermediate mobility, representing actin and tropomyosin, as well as the components of myosin. The myosin preparation also contained trace amounts of actin and tropomyosin, which could be seen in heavily loaded gels such as the one shown in plate 11.

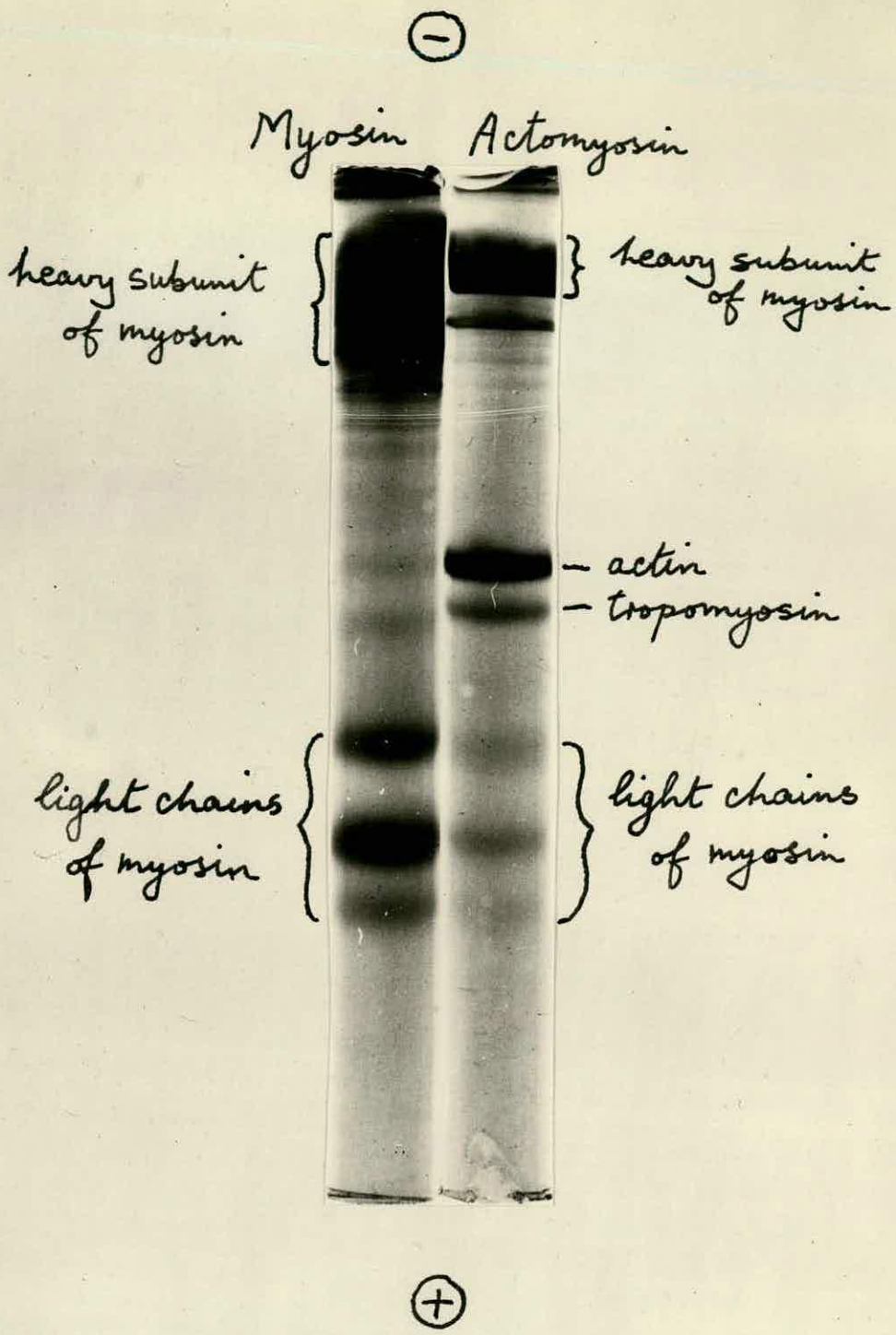
⊖
Myosin
Actomyosin
heavy subunit of myosin
heavy subunit of myosin
actin
tropomyosin
light chains of myosin
light chains of myosin
⊕

Plate II.

Preparation and assay of antisera to myosin and actomyosin

Rabbits were immunised by the injection of suspensions of purified myosin or actomyosin. Appropriate volumes of the protein preparations in 50% glycerol were diluted in 0.5M CKl (without dithiothreitol) and the proteins were then precipitated by the addition of 14 volumes of cold water (without dithiothreitol). The precipitates were centrifuged into pellets and then resuspended in a small volume of the supernatant for injection.

The first injection was given subcutaneously and consisted of 10 mg of protein mixed with a volume of Freund's adjuvant (Difco) equal to the volume of the protein suspension. Two courses of injections into the ear vein were then given 30 and 60 days later and the rabbits were bled from the ear vein 9 days after each course. A course of injections consisted of 3 lots of 10 mg of protein administered on alternate days. Serum was collected after clot formation and stored at -20°C.

Antibodies were detected by Ouchterlony's double diffusion procedure in agar gels. 1% solutions (w/v) of agar (Oxoid 'ionagar' no.2) in 0.5M KCl, 0.02M tris pH7.3 were poured onto glass slides and allowed to solidify in thin films. Wells and troughs were cut and the antigen and antibody preparations were

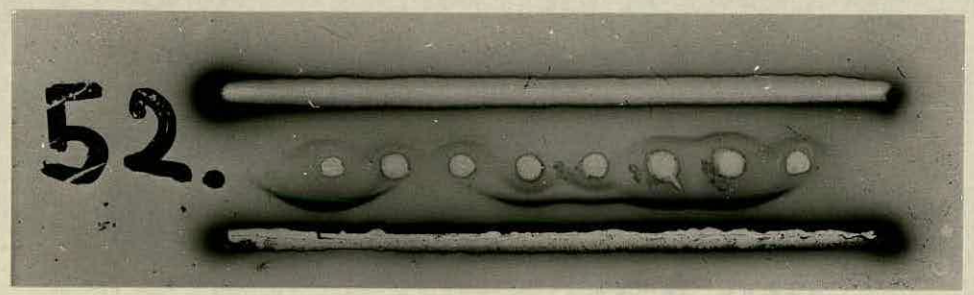

Plate 12.

placed into them from finely drawn out pasteur pipettes. Reactions were run at room temperature for 1-2 days. When the precipitin lines had appeared, the slides were washed for 12 hours in 0.5M KCl then for 3 hours in 0.15M NaCl, dried and stained with a 0.1% solution of Napthalene Black (Gurr) made up in mixture of 7 parts ethanol to 2 parts water to 1 part glacial acetic acid.

Plate 12 shows an Ouchterlony gel containing 8 wells and 2 troughs. The wells were cut with their centres 5 mm apart and 5 mm from the edge of the troughs. The top trough contained rat myosin prepared by N.S.T. Thomas according to Finck's method at 5 mg per ml and the bottom trough contained chicken actomyosin at 2.5 mg per ml. Numbering from the left,

wells 1,6 and 7 contained pure antiserum prepared against chicken actomyosin

wells 4 and 5 contained antiserum prepared against chicken myosin and

wells 2,3 and 8 contained antiserum prepared against rat myosin.

Strong lines of precipitation can be seen between the lower trough that contained the chicken actomyosin and the wells that contained antisera prepared against chicken myosin or chicken actomyosin. This shows that antibodies to the protein preparations had been evoked from the rabbits. Similarly, weak

lines of precipitation can be seen between the upper trough which contained the preparation of rat myosin and the wells (nos 2,3 & 8) which contained the antiserum raised against this preparation. Weak lines of precipitation can also be seen between the anti-chicken actomyosin antiserum (wells 1,6 & 7) and the rat myosin preparation. However, no precipitation can be seen between the anti-chicken myosin antiserum (wells 4 & 5) and the rat myosin preparation. This is in accordance with Finck's demonstration (1965b) that the cross reactivity of chicken and rabbit myosins decreases as the myosin preparations are increasingly purified. Further work would need to be done to confirm the findings for rat myosin, since electrophoretic analysis of the particular preparation of rat myosin used suggested that the proteins had become partially degraded.

CHAPTER III

Quantal mitosis in myogenesis

Introduction

It has been proposed for several types of differentiating cell that the terminally differentiated cell is derived from a proliferating precursor at a 'quantal mitosis' which differs in some way from ordinary proliferative mitoses. The maturation of the epithelial cells of the mammary gland (see page 8) provide an example of the coupling of differentiation and proliferation. Myogenic cells are also widely considered to undergo 'quantal mitoses'. The chief proponent of the quantal mitosis theory for myogenesis has been Holtzer (e.g. Holtzer and Sanger 1970). This author and co-workers have postulated that there are two classes of mononucleate cells involved in myogenesis, presumptive myoblasts which multiply, and myoblasts which are post-mitotic, fuse into synctia and accumulate the contractile proteins. Myoblasts are supposed to be derived from presumptive myoblasts at a 'quantal mitosis' after which one or both daughter cells is a post-mitotic myoblast. This theory predicts that a myogenic cell cannot at one time be capable of the two alternatives: to divide, or to fuse into a myotube without further division. The evidence on which the quantal mitosis theory for

myogenic cells is based comes from the autoradiographic study of DNA synthesis in differentiating cultures of chick myogenic cells. In these cultures proliferating cells were found to spend an average period of 2 hours in G1 before beginning the next round of DNA synthesis, whereas cells which fused into myotubes spent a minimum time of 5 hours between the end of the last mitosis and the time at which they were first found in syncytia (Bischoff & Holtzer, 1969). From this it appeared that the withdrawal of the cells from the mitotic cycle precedes the initiation of fusion and it was proposed that the withdrawal takes place at the mitosis preceding fusion, though it was not specified during which part of the mitosis the critical events take place which turn an ordinary mitosis into a quantal mitosis. If the tissue culture environment can be manipulated so that cultured cells will multiply or differentiate according to the conditions acting upon them, then it is possible to test the quantal mitosis theory by investigating whether cells which fuse into myotubes must first undergo a division in the presence of the culture conditions which cause them to differentiate, or whether they can be switched from the mitotic cycle to the post-mitotic state without going through a ('quantal') mitosis.

The cells of the L_5 myogenic cell line can be switched from proliferation to differentiation if the

concentration of the foetal bovine serum in the medium is lowered (see page 24). When small clonal patches of these cells are grown on a dish in medium containing 10% foetal bovine serum and on the 6th day the medium is replaced with medium containing only 1% of serum, myotube formation will occur in many of the clones within a further 5 days. If, however, the cells are fed with 10% serum on the 6th day they will continue to multiply and within 5 days no myotubes will appear. What permits the mononucleate cells to differentiate in 1% serum or what prevents them from doing so in 10% serum are unknown, but the quantal mitosis theory predicts that the cells must undergo a cell division in the permissive 1% serum before they can differentiate. Labelling experiments were performed to investigate whether they fulfil this prediction.

Materials and Methods

Cell Culture.

A monodisperse suspension of L_5 cells was plated on several dishes at a dilution appropriate to produce sparsely distributed clones (see page 24) and the cells were cultured in 10% serum for 6 days. On the 6th day some of the dishes were fed with 10% serum and some with 1% serum and tritiated thymidine (0.3 Ci/ml) was added to the medium. After 5 days in the thymidine-containing medium the cultures were

rinsed with Dulbecco A and fixed in cold methanol. Autoradiographs were prepared from the whole cultures.

Autoradiography

Cultures incubated in medium containing tritiated thymidine were fixed in cold methanol for a minimum of 30 minutes and dried. The dishes were treated with 5% trichloroacetic acid at 0°C for 5 minutes, washed in water for 1 hour to remove unbound nucleotides (Ringertz et al 1969) and then dried again. Ilford L_4 emulsion was diluted 1:1 by weight with distilled water and heated to 37°C. It was poured onto the dishes and immediately drained off. The film of emulsion that remained was dried at 18°C in a current of air and exposed to the radioactive cells that it covered for 7 days at 4°C. The autoradiographs were developed with D19B (Kodak) for 4½ minutes at 18°C and fixed in Fix-sol (Johnson) diluted 1:5 for 20 minutes.

Thymidine (methyl H^3) was obtained from the Radiochemical Centre, Amersham, at a specific activity of 20.4 Ci/mmol. It was found that when the isotope was added to culture medium to make a final concentration of 0.3 Ci/ml, the nuclei of L_5 cells became labelled with autoradiographic grains if proliferating cells were exposed to this medium for as little as 9 minutes and then treated according to the above procedure.

Staining

Cultures and autoradiographs were stained with Giemsa R66 (Gurr). 6 cm dishes were filled with 0.5 ml of Giemsa diluted in 10 ml of Giemsa buffer pH 6.8 (Gurr), stained for 40 minutes, rinsed in distilled water for 5 minutes and dried. Improved staining of autoradiographs was obtained if the emulsion was pretreated with the following solutions and then dried before being stained in the ordinary way. Each solution was left on the dishes for 3 minutes.

1. Buffer pH 6.8 and 50% alcohol mixed 1:1
2. 95% alcohol
3. Buffer pH 6.8
4. Buffer pH 6.8 and 50% alcohol mixed 1:1.
5. 95% alcohol
6. Buffer pH 6.8
7. Buffer pH 6.8

Results and Discussion

Autoradiographs were prepared from cultures of clones after 5 days in medium containing 1% serum and tritiated thymidine. All the clones that had been cultured in this way were found to contain many nuclei that were unlabelled with autoradiographic grains. In all the clones where extensive differentiation had taken place some of these unlabelled nuclei had become incorporated into myotubes (see

Plate 13. Autoradiograph prepared from a 6-day clone of L_5 cells cultured for 5 days in medium containing 1% serum and tritiated thymidine.

Zeiss photomicroscope II x35

Plate 14. as above x200

plates 13 and 14). This shows that some of the cells in the clones (at 6 days after plating) are capable of undergoing differentiation without undergoing detectable DNA synthesis. If there is such a thing as a quantal mitosis then these cells must already have undergone the DNA synthesis phase of this mitosis at the time that the 1% serum was added. Parallel dishes of cells fed with 10% serum on the 6th day did not show any differentiation in the subsequent 5 days, but nonetheless may have contained post-mitotic cells which were merely prevented from fusing into syncytia by the higher concentration of the serum. In order to test this possibility the dishes which were fed with 10% serum on the 6th day were given tritiated thymidine at the same time. At the time the cells were fixed (on the 11th day), there were more nuclei in the clones fed with 10% serum on the 6th day than in those fed with 1% serum, but all the clones were descended from similar initial (6 day) populations. Are there enough cells by the 6th day which do not synthesise DNA again, even in 10% serum, to account for the number of unlabelled nuclei that appear inside myotubes in 1% serum? The autoradiographs from a typical experiment indicate that there are not:-

Number of unlabelled nuclei within syncytia in 40 (6 day) clones cultured 5 further days in 1% serum with H^3-thymidine	402 (average 10 per clone)
Total number of unlabelled nuclei in 40 (6 day) clones cultured 5 further days in 10% serum with H^3-thymidine	120 (average 3 per clone)

(Since binucleate cells can arise in tissue culture without cell fusion, for the purpose of scoring unlabelled nuclei in myotubes, a syncytium was defined as a cell containing 3 or more nuclei.)

This result shows that at 6 days there were more cells able, in appropriate circumstances, to undergo myogenic cell fusion without undergoing detectable DNA synthesis than there were cells already withdrawn from the mitotic cycle. The quantal mitosis theory would have predicted that there would have been at least as many unlabelled cells in the clones in 10% serum as there were unlabelled nuclei inside myotubes in the 1% serum. The results indicate that at least some of the cells could at one time (on the 6th day) be capable of either undergoing the DNA synthetic phase of a mitosis or of becoming incorporated into a myotube without first undergoing detectable DNA synthesis.

Is it possible that the unlabelled nuclei inside myotubes were derived from cells which were

caught at the end of the S period or in G2 when the medium was changed to 1% serum? The isotope concentration and autoradiographic conditions used produce some nuclei which would be scored as having been in S when proliferating L_5 cells are exposed to the thymidine for as little as 9 minutes, so if it is assumed that thymidine incorporation is smooth throughout S, as is generally the case for mammalian cells (Mitchison 1971), then only short periods will have gone undetected. It is not possible by the technique of thymidine labelling to distinguish the nuclei of cells which are caught by the 1% serum in G1 and fuse into myotubes from those which are caught in G2 and go through this phase and mitosis and then fuse since both will be unlabelled. Although the present evidence against the quantal mitosis theory for the L_5 myogenic cell line therefore only extends to showing that the cells do not need to undergo a round of DNA synthesis in the medium which enables them to differentiate before they can permanently withdraw from the mitotic cycle, it seems quite likely that cells can withdraw without going through any part of a mitosis in the permissive medium.

A recent report claims to show that the quantal mitosis theory is also invalid for myogenic chicken cells. It was found that cultures, which would otherwise have ceased proliferating and would have

differentiated, could be stimulated to more DNA synthesis (measured by the radio-activity found in extracted DNA after a tritiated thymidine pulse) if they were given fresh medium (O'Neill & Stockdale, 1972). Work on newly explanted rat cells has shown that when cultures that are undergoing cell division and differentiation are subjected to treatments which are known to block DNA synthesis (2mM-thymidine or 5000r of X-rays) then differentiation is also inhibited. This has been taken to show that a round of DNA synthesis is necessary for differentiation (Yaffe, 1971). It is possible that this interpretation is correct and that the L_5 cell line behaves differently in this respect from newly explanted cells. Yaffe's results could, however, also be explained by the fact that his culture conditions probably stimulated cell division. It is difficult to interpret the behaviour of cells which are both stimulated to divide and prevented from doing so. 2mM thymidine prevents DNA synthesis and when it is added to proliferating cultures the cells caught in the middle of S remain there while the other cells collect at the G1/S boundary. X-rays interfere with DNA synthesis by breaking DNA strands and therefore also probably block cells in the S period. Since it is myoblasts that are in G1 which withdraw from the mitotic cycle and fuse into myotubes, it might be expected that

cells blocked at the beginning or in the middle of S do not differentiate. In mitogenic culture conditions, where DNA synthesis is also blocked, a large proportion of the myoblasts may be in this condition. That differentiation does not take place when myoblasts are stimulated to divide and then blocked does not mean that culture conditions, such as reduced serum concentration, could not be devised which would not stimulate cell division but which would allow differentiation. The culture conditions usually employed for the study of myogenesis support proliferation as well as differentiation and this fact may well have been a source of confusion about a possible role of mitosis in myogenic differentiation *in vitro*. Just because cells can divide in a differentiating culture it does not follow that they must do so.

Work on myogenesis *in vitro* involves the terminal stages of differentiation. Mitosis may have a critical role at earlier stages such as during the determination of the myogenic cell type. However, the investigation of the function of mitosis at any stage in differentiation is likely to be hampered by the fact that cell division for tissue growth is an integral part of normal embryogenesis.

CHAPTER IV

The role of cell fusion in myogenesis

Introduction

It has been shown that cell fusion is an essential step in the differentiation of rat and chicken myogenic cells *in vitro*. If the calcium concentration in the medium is reduced, the process of cell fusion is held in check and the proteins characteristic of the differentiated muscle cell are also prevented from appearing. When calcium is added to the cultures to bring its concentration back to the normal level, the cells differentiate to form normal myotubes (Shainberg et al 1969; Paterson and Strohman 1972). Cells can be induced to fuse into syncytia by the use of Sendai virus (Okada 1972), and pretreatment of the virus with ultraviolet light can prevent its subsequent replication in the cells (Harris et al 1966). This technique provides a means to investigate the role of cell fusion in myogenesis. Can myoblasts which are fused together by the effect of inactivated Sendai virus undergo normal differentiation in response to the artificial cell fusion? Virus-induced fusion can also be used to study myoblasts which have lost the capacity to undergo normal myogenic cell fusion.

Failed myoblasts ($L_{5.1.1.5}f_1$ see page 31) have been fused together with Sendai virus in order

to determine whether they can be induced to differentiate by this means. Ordinary proliferating L_5 myoblasts, free of differentiated myotubes, have also been fused into syncytia artifically and the effect of this premature cell fusion on the subsequent differentiation of the cells has been followed. As a control for these experiments ordinary myoblasts were subjected to virus-induced cell fusion after pre-treatment in culture conditions designed to promote myogenesis but in which normal myogenic cell fusion was held in check by calcium deficient medium.

Materials and methods

Virus-induced formation of syncytia

Large syncytia were formed when concentrated suspensions of L_5 cells were treated with inactivated Sendai virus, but many of the cells were killed if too much virus was used. The optimal ratio of cells to virus to produce many syncytia and very few dead cells was determined by trial and error. The procedure in a typical syncytium-making experiment was as follows: L_5 cells from confluent monolayers covering six 9 cm dishes or their equivalent, were suspended and dispersed in 0.25% trypsin solution in Dulbecco A. The trypsin was removed by centrifugation (200g 5 mins) and the cells were rinsed in two changes of Dulbecco A. They were then suspended in Hanks'

solution (made up without glucose) and centrifuged once more. The pellet was suspended in a total of 0.3 ml of Hanks' solution and 0.1 ml of Hanks', containing 1500 HAU of inactivated Sendai virus, was added and mixed in. The mixture was chilled in ice for 15 minutes, shaken in a water bath at 37°C for a further 15 minutes and then gently, by means of a wide-nozzled pipette, diluted in whole growth medium and put onto new tissue culture dishes. A dilution factor was normally used to leave the cells at $\frac{1}{4}$ of their density in the original monolayers.

Prevention of myotube formation with calcium deficient medium

It has been shown that newly explanted rat myoblasts can be prevented from differentiating if the concentration of $CaCl_2$ in the medium is reduced to 35μM, although the capacity of the cells to proliferate is not much affected by this treatment (Shainberg et al 1969). Since this work involved medium containing 10% serum, the serum was dialysed against Ca^{++} free saline in order to bring the final Ca^{++} concentration in the medium down to 35μM. As only 2% serum is used for differentiating cultures of L_5 cells, the serum was not dialysed, but instead calcium free medium containing 2% of ordinary serum was prepared and varying amounts of $CaCl_2$ were then added to this medium from zero to the normal level (1260μM). Confluent monolayers of L_5 cells which were on the

point of differentiation were then cultured in the different levels of calcium to determine the highest concentration at which differentiation was still prevented. If no $CaCl_2$ was added the cells appeared unhealthy. Cells cultured in higher and higher concentrations of calcium achieved more and more differentiation. It was found that in medium containing 8μM-$CaCl_2$ and 2% serum, differentiation was very largely suppressed although the cells appeared to survive this treatment and a few small myotubes still formed. Medium of this composition was used as 'calcium deficient medium'.

Immunofluorescent detection of contractile proteins.

The accumulation of contractile proteins in differentiated muscle cells was detected by the indirect immune fluorescence technique using a rabbit antiserum raised against a preparation of actomyosin (see page 41). This antiserum was prepared against chicken contractile proteins, but it was found to cross react with rat antigens and to bind to the myotubes in differentiated cultures of L_5 cells. The procedure used for indirect immune fluorescence was as follows: a dish of cells was rinsed three times in Dulbecco A, drained very thoroughly and dried. The area of the dish to be investigated was encircled

with pencil wax (Royal Sovereign Chinagraph no.480). A drop of anti-actomyosin antiserum, prediluted to $^{1}/_{50}$ in Dulbecco A, was then placed on the area, where it was contained by surface tension within the circle of wax. The dish was left at room temperature in a damp chamber for a minimum of 30 minutes. The antiserum was then rinsed off and the whole dish was washed in a large volume of agitated Dulbecco A for 30 minutes with two changes, drained and dried. Fluorescein-labelled anti-rabbit immunoglobulin sheep antibodies (Wellcome laboratories) were diluted to $^{1}/_{10}$ in Dulbecco A and a drop was placed inside the the wax circle, allowed to bind for 30 minutes and then rinsed off as before. The cells were finally mounted in a solution of 90% glycerol in a $NaHCO_3$ buffer pH8-9 and viewed with a Zeiss photomicroscope II using epi-illumination, a BG12 exciter filter, F1 500 reflector and a Zeiss barrier filter no.53.

A number of procedures for fixing cell cultures for immunofluorescence were tried and drying without added fixative was found to be the most successful. It has been reported that cells detach easily from the substratum if they are merely dried for fluorescence work (Dawkins and Lamont 1971), but this was not found to be a problem with the particular combination of dish and cell used for the present experiments.

Measurement of creatine phosphokinase activity

Creatine phosphokinase (CPK; E.C.2.7.3.2) activity in cell extracts was measured by the method of Oliver (1955), using test kits obtained from Boehringer Mannheim. ATP formation from ADP and creatine phosphate is enzymatically coupled via hexokinase and glucose-6-phosphate dehydrogenase to the reduction of NADP. The conversion of NADP to NADPH is measured by the increase in absorbancy at 366nm. Concentrations in the test volume were:-

0.1 M triethanolamin buffer pH 7.0	0.6mM NADP
20 mM glucose	35 mM creatine phosphate
10 mM Mg acetate	>50 µg hexokinase
1.0mM ADP	>25 µg glucose-6-phosphate dehydrogenase
10 mM AMP	9.0mM glutathione

Cells were scraped off dishes in ice cold Dulbecco A with a rubber policeman and collected by centrifugation at 700g for 10 minutes. They were then resuspended in 1 ml of Dulbecco A and disrupted with a Dawe Soniprobe sonicator type 1130/1A for a total of 30 seconds with 1 minute breaks for cooling on ice after every 10 seconds. The suspension of sonicated cells was then centrifuged at 2000g for 10 minutes and 0.1 ml of the supernatant was mixed with 2.5 ml of the CPK assay mixture. The remaining supernatant and the pellet

were kept for DNA estimation. The increase in absorbancy at 366 nm was followed against an air blank in a 1cm silica cuvette at room temperature in a Beckman DB spectrophotometer. Readings taken at 1 minute intervals were plotted and the CPK activity in milliunits per ml of cell extract was calculated from the rate of increase of absorbancy (ΔE/minute) between the 5th and 10th minutes by reference to standard figures supplied with the test kits. After preliminary experiments, the number of dishes used for making each extract was adjusted to give a CPK activity per ml which fell within a readable range (8-240 milliunits; ΔE/minute 0.001-0.030).

Measurement of DNA

DNA in the sonicated cell samples was measured using the diphenylamine reaction (Burton 1956). The reaction results in the formation of a blue colour when DNA is heated with diphenylamine in acid solution. The DNA was first separated from the RNA and the protein by a modification of the procedures of Fleck and Munro (1962) and Ogur and Rosen (1950) devised by H.A.John (John 1966).

Acid insoluble components were precipitated from the material remaining after the measurement of the CPK activity by adding 2 ml of cold 0.2N-perchloric acid (PCA) and centrifuging at 2000g for 10 minutes.

The precipitates were rinsed twice with 0.2N-PCA. RNA in the pellets was hydrolysed with 4 ml of 0.3N-KOH at 37°C for 1 hour. After chilling to 0°C the acid insoluble components were reprecipitated with 3 ml of 0.6N-PCA and pelleted. DNA was extracted twice from the pellets with 0.5N-PCA at 70°C for 15 minutes using a total of 7 ml. 2 ml of the acid extract was mixed with 4 ml diphenylamine reagent and incubated at 28°C for 20 hours. Diphenylamine reagent was prepared immediately before use by adding 0.01 ml acetaldehyde solution (16 mg/1.0 ml water) to 20 ml diphenylamine solution (1.5 gm diphenylamine:1.5 ml H_2SO_4:100 ml acetic acid).

The intensity of the blue colour was read at 600 nm against a control assay to which 2 ml of 0.5 N-PCA had been added instead of acid extract. DNA concentrations were calculated from a curve prepared using salmon DNA dissolved in 0.5N-PCA at known concentrations and subjected to the diphenylamine reaction procedure.

Results and discussion

Morphology of artificial syncytia

Cultures of failed and ordinary L_5 myoblasts were grown and artificial syncytia were made from both types of cell. When ordinary L_5 myoblasts were used the syncytia were prepared from proliferating

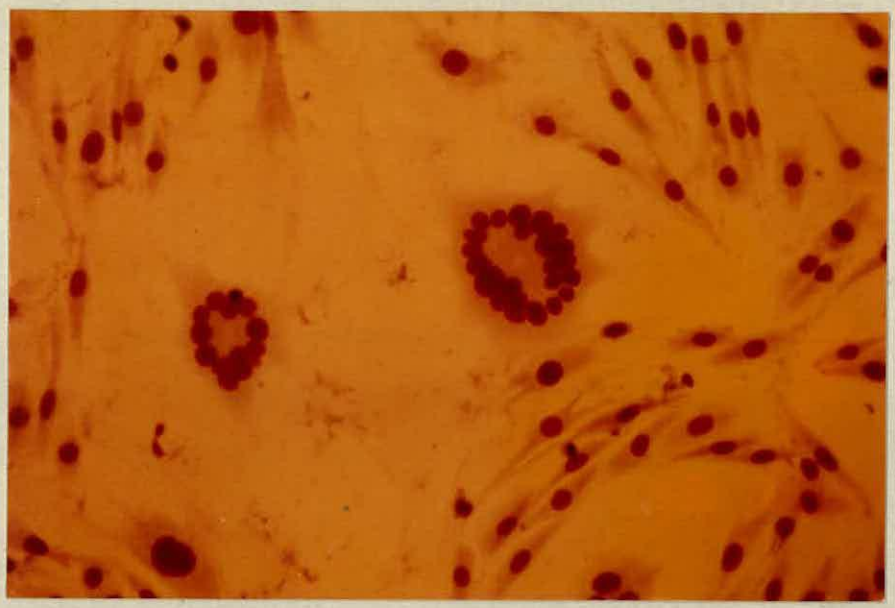

Plate 15. Artificial syncytia prepared from L_5 myoblasts. x150

Plate 16. Ordinary L_5 myotubes. x100

cultures before any naturally occurring cell fusion had taken place. After virus-induced fusion the syncytia were cultured in medium containing 2% serum, which promotes differentiation in normal cultures of L_5 myoblasts (see page 24). Whether the cells used were failed myoblasts or ordinary ones, the syncytia did not appear to organise themselves into myotube-like structures. They remained rounder than naturally formed syncytia and the nuclei tended to become grouped together in the middle of the cells (see plates 15 &16). When failed myoblasts were used the syncytia remained rounded and the cultures were slowly overgrown by mononucleate cells. When ordinary myoblasts were used, naturally forming myotubes began to appear after 4-5 days but even after 7 days the rounded artificial syncytia could be distinguished from the myotubes. That the differentiated myotubes had arisen by natural cell fusion from mononucleate cells in the culture, and not by conversion of the rounded artificial syncytia to the normal differentiated form, was deduced from the fact that these myotubes were at first relatively small compared to the artificial syncytia and also from the finding that L_5 cells put through a sham fusion procedure without the addition of virus, and diluted before further culture to a density equivalent to that of the virus treated cells, also began to form myotubes after 4-5 days.

In order to interpret the failure of syncytia formed by virus-induced cell fusion to organise themselves into myotubes, it is necessary to consider the ontogeny of ordinary myotubes. Myoblasts normally fuse together end to end (see plate 6) and time-lapse cinematography shows that the orientation of myoblasts in culture, as they align together before fusing, is reflected in the orientation of the myotube to which they give rise (K.W.Jones personal communication). It may be that the organisation of a myotube merely reflects the way that the myoblasts originally came together. Two studies indicate that this is not the case. Rounded 'disorganised' syncytia are formed by natural cell fusion if chicken myoblasts are cultured in a special 'non-filterable' fraction of embryo extract. When the 'filterable' fraction is later added to the culture medium, myotubes grow out from the globular syncytia (de la Haba and Amundsen 1972). Globular syncytia are also formed when chicken myotubes are treated with 10^{-6}M colchicine. When the colchicine is removed, nucleated sarcoplasmic processes grow out from the syncytia (Bischoff and Holtzer 1968). It therefore appears that rounded myogenic syncytia can organise themselves into elongated myotubes and the failure of artificial syncytia to do so requires an explanation.

The action of colchicine on myotubes had been correlated with the disassembly of microtubules (Ishikawa et al 1968). Examination of the ultrastructure of the artificial syncytia might show to what extent the assembly of microtubules characteristic of myotube morphogenesis can take place in these cells. The fact that the nuclei are grouped in the centre of the syncytia could be interpreted to mean that the cytoplasm does not have an extensive microtubular organisation. The cell membranes of the syncytia appear to adhere strongly to the dishes and in the absence of any resistance from microtubular organisation in the cytoplasm this might be expected to push the nuclei into the centre.

Accumulation of contractile proteins in artificial syncytia

Artificial syncytia made from failed myoblasts were now cultured in 2% serum and after various periods sample dishes were dried and tested for the presence of actomyosin by the immunofluorescence procedure. No positive fluorescence above the general background level shown by the mononucleate cells was found in the syncytia at any stage. This shows that the accumulation of the contractile proteins characteristic of normal myogenic syncytia is not induced when failed myoblasts are artificially fused together.

Plate 17.

The results with ordinary proliferating myoblasts were not so clear cut because cultures of artificial syncytia became contaminated by myotubes formed by natural cell fusion. Nevertheless, the syncytia formed by virus-induced cell fusion of proliferating L_5 cells did not appear to accumulate the antigens associated with ordinary differentiation. Small myotubes, probably formed by natural cell fusion (see above), had begun to appear 4 days after the fusion procedure and in cultures dried down at this time and tested for the accumulation of acto-myosin by the immunofluorescence procedure, these myotubes could be seen to be brightly fluorescent against a background of many negative syncytia (see plate 17).

Since it is possible that artificial syncytia do not exhibit normal myogenic differentiation because the myoblasts have not undergone appropriate developmental changes which precede natural cell fusion, an attempt was made to collect the myoblasts, before subjecting them to virus-induced fusion, at the stage in normal differentiation which immediately precedes fusion. Confluent L_5 myoblasts were cultured in calcium deficient medium containing 2% serum (see materials and methods) for 6 days, by which time a dish cultured in parallel in ordinary medium containing 2% serum had undergone extensive

differentiation. The cells were then subjected to the virus-induced fusion procedure and cultured for a further 7 days in calcium deficient medium. At the end of this period most of the syncytia were found to have the round shape characteristic of artificial syncytia. These syncytia did not show fluorescence after the usual procedures. Some brightly fluorescing myotubes were also found to be present, but the origin of these was uncertain because the calcium deficient medium used did not completely suppress natural myotube formation.

Creatine phosphokinase (CPK) activity in cultures containing artificial syncytia

Since pure cultures of artificial syncytia were not obtained free of myotubes formed by natural cell fusion in the experiments with ordinary myoblasts, a method such as the immunofluorescence technique, which detected differentiation in individual cells, had limitations as a means of investigating the effect of virus-induced syncytium formation. A technique was required which would give a quantitative measure of differentiation in the entire culture. The level of creatine phosphokinase, a muscle-associated enzyme, has been shown to increase in cultures of a rat myogenic cell line when the cells differentiate (Shainberg et al

1971). The elevation of the level of this enzyme has also been shown to be held in check if myotube formation is inhibited in cultures of newly explanted rat myoblasts by calcium deficient medium (Shainberg et al 1969). This enzyme can therefore provide a measure of myogenic differentiation in cultures of artificial syncytia. Because the activity of the enzyme only rises slowly in differentiated L_5 cells, the syncytia must be maintained for several days in order to determine the effect of the virus-induced cell fusion. In fusion experiments with ordinary myoblasts, therefore, calcium deficient medium must be used for this period in order to hold natural cell fusion and differentiation in check.

Failed myoblasts were subjected to the virus-induced fusion procedure and cultured in 2% serum. The level of CPK in the cultures was determined after 18 days. Cells which had been subjected to a sham fusion procedure, in which Hanks' solution was added to the fusion mixture instead of a suspension of Sendai virus, were cultured and assayed in parallel as a control. CPK in proliferating failed myoblasts cultured in 10% serum was also measured. The enzyme activity was expressed as a function of the DNA content in the samples.

CPK was not measured in syncytia prepared from proliferating ordinary myoblasts but experi-

ments were done with mature cultures of ordinary myoblasts prevented from differentiating normally by calcium deficient medium. Additional assays as controls for possible deleterious effects of this medium were made as follows: confluent monolayers of L_5 cells were cultured for 6 days in calcium deficient medium (8 μM $CaCl_2$) containing 2% serum and then split into equal samples. Half of the cells were subjected to the virus-induced fusion procedure and half to a sham fusion procedure. The samples were then split again and half of each was cultured in normal medium with 2% serum and half in calcium deficient medium with 2% serum. Calcium is necessary for the virus-induced cell fusion process (Okada and Murayama 1966) but the cell fusion reaction was performed in such a small volume that the amount of additional calcium added to the culture medium from the fusion mixture, when the cells had been diluted to their final plating density was not significant. After 13 days the cultures were assayed for CPK activity. The results of the assays of the cultures of failed myoblasts and ordinary myoblasts are shown in table 2 .

Other work with L_5 cells has shown that they can exhibit a five fold increase in CPK activity (58-277 milliunits/10μg DNA), when they

TABLE 2

Failed myoblasts ($L_{5.1.1.5^{f}1}$)

	ΔE/min	CPK milliunits per 4 dishes	DNA μg per 4 dishes	milliunits CPK per 10μg DNA
A. Proliferating cells; 10% serum	0.0049	39.2	18	21.8
B. Sham fusion; cultured 18 days in 2% serum	0.0244	192.2	70	27.4
C. Virus-induced fusion; cultured 18 days in 2% serum	0.0188	148.4	53	27.9

Ordinary myoblasts ($L_{5.1.1.5.5.2.1.9.2.8}$) previously cultured for 6 days in 2% serum in calcium deficient medium

	Culture medium (containing 2% serum) for 13 days subsequent to fusion procedure	ΔE/min	CPK milliunits per 4 dishes	DNA μg per 4 dishes	milliunits CPK per 10μg DNA
D. Sham fusion	1260μM $CaCl_2$	.0062	48.6	16	30.4
E. Sham fusion	8μM $CaCl_2$	.0108	85.4	25	34.2
F. Virus-induced fusion	1260μM $CaCl_2$	.0064	50.2	13	38.6
G. Virus-induced fusion	8μM $CaCl_2$	.0048	38.4	15	25.6

differentiate (see page 91). In relation to that finding the figures in table 2 do not show any significant changes in CPK in the cells. In the failed myoblasts the enzyme activity per dish increased over prolonged culture but this increase can be accounted for by the increase in cell number which took place and which is reflected in the higher DNA readings. It is probably the proliferating mononucleate cells which are responsible for the increase in DNA levels and the presence of syncytia may therefore account for the fact that the DNA reading for sample C is lower than that for B. There is no evidence in these data for any effect of stimulation by virus-induced cell fusion on the CPK levels in the failed myoblasts. Together with the fact that the antigens associated with differentiated myotubes were not found in similar artificially constructed syncytia, this could be interpreted to mean that the failed myoblasts may have undergone a change which affects their capacity to differentiate at a stage earlier than myogenic cell fusion. However, the results of the experiments on artificial syncytia made from ordinary myoblasts cast doubt on this explanation.

For reasons already given the immunofluorescence technique did not give a conclusive picture of the behaviour of ordinary myoblasts after virus-induced cell fusion. The quantitative measure

of differentiation which the CPK assays provide is therefore of particular interest. A comparison of the CPK activity per unit of DNA from samples E and G should show whether virus-induced cell fusion can stimulate CPK activity in the cells. No such stimulation was found and in fact a lower reading was obtained from the virus-treated cells. This suggests that virus-induced cell fusion does not even stimulate differentiation by ordinary myoblasts. However, the control cells cultured in normal medium after the sham fusion procedure (sample D) did not attain an elevated CPK level. This calls into question the validity of the whole experiment with calcium deficient medium. Differentiated cultures of L_5 cells can reach values of 270 milliunits of CPK per 10μg DNA and cultures of proliferating L_5 myoblasts give values of 50-60 milliunits (see page 91). The reason for the abnormally low levels for all the samples D-G could be some deleterious effect of the 6 day pretreatment in calcium deficient medium. This explanation is consistent with the observation that the cultures used for sample D were not so extensively differentiated into myotubes as L_5 cells normally are after 13 days in 2% serum.

DNA synthesis in artificial syncytia

The reason for differences between the behaviour of naturally formed myotubes and

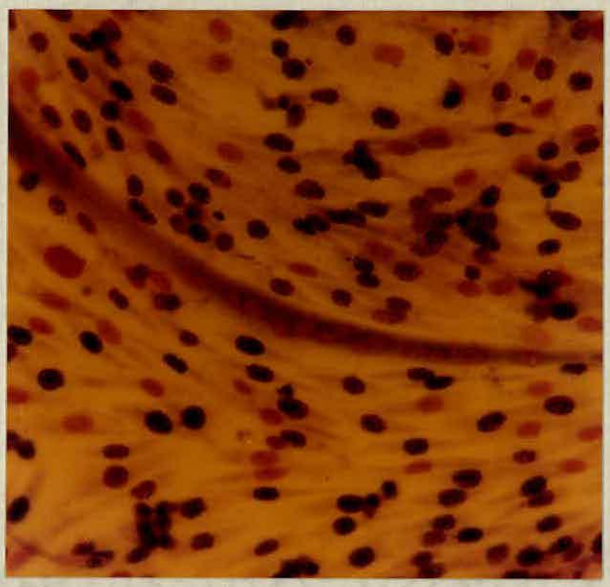

Plate 18. x 150

and artificially prepared syncytia could be that natural cell fusion is a selective process, as has been shown by various techniques, whereas virus-induced fusion probably involves a heterogeneous collection of cells. It has been shown with differentiating chick myoblasts that cells undergoing fusion are always in G1 (Strehler et al 1963) and that once a nucleus is incorporated into a myotube it does not synthesise DNA again (Bischoff & Holtzer 1969). The latter finding has been confirmed for cells of the L_5 line. Cultures containing differentiated myotubes were fed with medium containing 10% serum to stimulate cell division (see page 24) and tritiated thymidine was added to the medium to label cells which were synthesising DNA. An area of one of the resulting autoradiographs, prepared by the usual method (page 48), is shown in plate 18. It can be seen that many mononucleate cells are labelled whereas intrasyncytial nuclei are not. Experiments of this sort showed that nuclei within myotubes of L_5 origin do not synthesise DNA.

By the immunofluorescence technique it was shown that the rounded syncytia formed from myoblasts by virus-induced cell fusion did not accumulate actomyosin. It can be argued that when

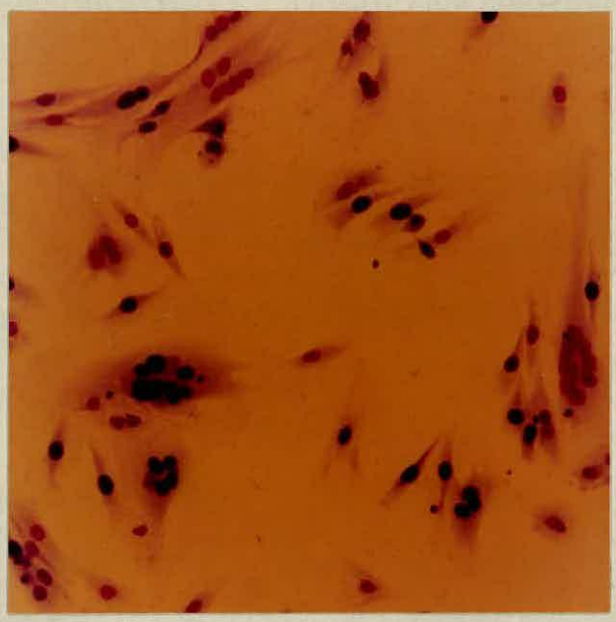

Plate 19. Autoradiograph prepared from a culture of artificial syncytia maintained in tritiated thymidine-containing medium for 12 hours from the time of virus- induced cell fusion. x100

Plate 20. Autoradiograph showing artificial syncytium containing mostly unlabelled nuclei after 4 days in tritiated thymidine-containing medium. x200

myoblasts are artificially fused, some of the cells are synthesising DNA or are in G2 and that this upsets the process of differentiation. Experiments were therefore conducted to investigate DNA synthesis in artificial syncytia. Cultures of ordinary proliferating L_5 myoblasts containing no myotubes were subjected to the usual virus-induced fusion procedure. Tritiated thymidine (0.3 Ci/ml) was added to the medium immediately after the procedure and autoradiographs were prepared from sample cultures fixed after 12 hours. It was found that many of the syncytia contained nuclei which were labelled with autoradiographic grains. This indicates that cells in the process of synthesising DNA are included in artificial syncytia and that the process of cell fusion does not itself immediately suppress DNA synthesis. The degree of labelling was not the same in the different nuclei within a syncytium and many syncytia contained some nuclei which were labelled and some unlabelled. This asynchronous behaviour is consistent with findings made on other cell types where DNA synthesis appears to be asynchronous within a newly formed multinucleate cell though for some cells it becomes co-ordinated and synchronous within 24 hours (Johnson & Harris 1969a). In order to investigate whether DNA synthesis took place in all the artificial syncytia in the experiments

with L_5 cells, cultures were continuously exposed to tritiated thymidine for 4 days from the time of cell fusion. After 4 days some of the intrasyncytial nuclei were unlabelled, but very few of the syncytia contained no labelled nuclei at all (see plate 20). If DNA synthesis by any of the nuclei in a syncytium is sufficient to upset normal differentiation then this could account for the failure to find differentiation in artificial syncytia.

General discussion

Natural cell fusion has been shown by the experiments with calcium deficient medium (Shainberg et al 1969) to be a necessary step in the progression of events by which myogenic cells are normally converted from proliferating myoblasts to mature myotubes. The attempts to simulate the natural process of myotube formation by virus-induced cell fusion have failed to show any convincing evidence that myogenic processes can be initiated by the process of syncytium formation alone. Since the ordinary myoblasts do not appear to differentiate when subjected to artificial cell fusion, it is not possible to conclude very much from the similar behaviour of failed myoblasts. The cells in a Sendai virus-treated culture of ordinary

myoblasts which do not undergo artificial fusion are not prevented from subsequently undergoing natural cell fusion and differentiation (see plate 17). It therefore seems unlikely that the dose of virus used in these experiments has a damaging effect on the cells. A more likely explanation of the failure of artificial syncytia to exhibit the characteristics of myotubes is that the cell fusion itself has upset the normal sequence of events in differentiation.

It is possible that the particular processes of membrane rearrangement involved in natural cell fusion have a special triggering effect on the succeeding events in myogenic differentiation. If this is the case, the artificial fusion of myoblasts may prevent myogenesis by interfering with the membrane rearrangement processes. There are some indications against this interpretation since myogenesis can under certain circumstances take place in cells which have not undergone fusion. First of all mononucleate cells occasionally differentiate in culture and accumulate contractile proteins while still containing a single nucleus (Okazaki & Holtzer 1965). The second line of evidence comes from experiments with cytochalasin B. This drug inhibits cell movement and cell division and when chick myogenic cells are cultured in the presence

of low concentrations of it (0.5μg/ml) many differentiated binucleate myotubes are formed (Sanger et al 1971). It is probable that these cells have undergone nuclear division without cytokinesis, in which case they have undergone the maturation events involved in differentiation without undergoing cell fusion.

An alternative explanation for the behaviour of artificial syncytia of myoblasts is that the mononucleate cells involved in the virus-induced cell fusion process have not all undergone covert developmental changes which may precede natural cell fusion. There is evidence from work with newly explanted rat cells that myoblasts undergo a process of maturation before cell fusion (Yaffe 1971). The process probably involves the accumulation of messenger RNA molecules appropriate for the differentiated cell. The evidence for this is that if myoblasts are treated with actinomycin D at a concentration which prevents all RNA synthesis they can still proceed to differentiate and attain elevated enzyme levels if the actinomycin D is administered up to 6 hours before cell fusion begins (Shainberg et al 1971). After cell fusion rat myotubes are rather resistant to actinomycin D compared to ordinary mononucleate myoblasts (Yaffe & Feldman 1964), and the incorporation of radioactive precursors into RNA is also significantly reduced (Yaffe & Fuchs 1967), so the

synthesis of a large proportion of the total RNA molecules appropriate to the differentiated cell may take place before cell fusion. If differentiation by L_5 cells involves similar processes and artificial cell fusion formation is premature for most of the cells incorporated into syncytia, then the syncytia may not contain sufficient complements of mRNA molecules for normal post-fusion differentiation. Premature fusion for even a small proportion of the cells contributing to a syncytium might also upset differentiation in a more subtle way. There may be factors in the cytoplasm of a proliferating cell which can counteract the normal myogenic progression of events if the cell is included in an artificial syncytium. This possibility could be further investigated by the virus-induced fusion of proliferating myoblasts to naturally formed myotubes.

The experiments with calcium deficient medium were designed to overcome the problems of the premature fusion of myoblasts. The results achieved so far with L_5 cells in calcium deficient medium have not been encouraging. Previously published work on the inhibition of natural cell fusion by calcium deficient medium involved newly explanted cells and the cell line may in some respects be less easy to manipulate. There are, however, two

good reasons for using a myogenic cell line for virus-induced fusion experiments. First, a cell line can provide pure samples of one cell type whereas primary cultures of myoblasts are always contaminated with fibroblasts. The second important reason is that cell lines are much more responsive to the fusing effects of Sendai virus. The fusion index, a measure of the proportion of the cells in a sample that undergo virus-induced fusion, varies according to the type of cells used and it has been found for several species and cell types that cell lines which have been grown in culture for a long time have higher fusion indices than newly explanted cells (Okada & Todokoro 1963). Preliminary experiments with newly explanted rat myogenic cultures indicated that this difference also applies to myoblasts. It might therefore be worthwhile to re-examine the principle of pretreatment in calcium deficient medium in order to obtain a means of synchronising myoblasts at the pre-fusion stage. The expression of the myogenic potentiality in myogenic cell lines is, however, notoriously sensitive to changes in the culture conditions (Richler & Yaffe 1970 and see page 34) and the results achieved so far with L_5 cells do not encourage this approach.

CHAPTER V

The control of differentiation in myoblast x fibroblast hybrid cells

Introduction

Work which has been done on hybrid cells has shown that, even within a single type of cross, some differentiated functions of one of the parents can be extinguished while others persist (see page 14). It is therefore of interest to know whether the capacity to express the many processes of myogenesis can be inherited by a myoblast x fibroblast hybrid cell. Myogenesis *in vitro* normally involves a sequence of processes, including the withdrawal of the myoblast from the mitotic cycle, cell fusion, the accumulation of contractile proteins and the attainment of elevated levels of various enzymes. A knowledge of whether different steps in the sequence can be expressed independently in the hybrid cells or whether the suppression of one step leads to the suppression of later ones may contribute to the understanding of the control of normal myogenesis. Proliferating hybrids were made between the L_5 myogenic cell line and the two L cell derivatives A_9 and B_{82} (Littlefield 1966). Two aspects of the biology of the hybrid cells

were then investigated, their ability to form myotubes, an early manifestation of myogenic differentiation and their capacity to attain elevated levels of the muscle-associated enzyme creatine phosphokinase which, in L_5 cultures, is expressed several days after cell fusion has begun.

Materials and Methods

Virus-induced cell fusion

Hybrid cells were made by the monolayer fusion technique (Davidson 1969). A typical procedure was as follows: cells from recently derived clones of the A_9 or B_{82} cell lines were mixed with cells of a clone from the L_5 myogenic cell line and allowed to settle together on a 6 cm dish for a minimum of 24 hours. The dish was then rinsed three times in Dulbecco A and 5 ml of medium without serum or antibiotics but containing 46,000 HAU of inactivated Sendai virus was added. The dish was chilled on an ice-covered tray (see page 102) for 30 minutes, rinsed twice in ice cold serum-free medium and then returned to the incubator in this medium for 2 hours. At the end of this period whole growth medium containing the components for the HAT selection technique (see below) was substituted, and after 24 hours the cells were

dispersed in trypsin solution and plated out at a low density in HAT medium so that individual clones could be distinguished after a few days.

HAT selection system

The system devised by Littlefield (1966) was employed by which cells deficient in thymidine kinase (TK) or inosinic acid pyrophosphorylase (IPP) can be selectively killed. The basis of the technique is that aminopterin inhibits dihydrofolate reductase which converts dihydrofolate to tetrahydrofolate. In the absence of the cofactor tetrahydrofolate the methylation of dUMP to dTMP by thymidylate synthetase, the de novo synthesis of purines and the conversion of serine to glycine by serine transhydromethylase are all blocked. When normal cells are treated with aminopterin they will proliferate if exogenous thymidine, hypoxanthine and glycine are present in the medium. Cells which lack TK or IPP activity cannot incorporate thymidine or hypoxanthine, respectively, and therefore die off. The four components to the selection system were added at the following molarities (Klebe et al 1970a):

Hypoxanthine	10^{-4}
Aminopterin or amethopterin	4.10^{-7}
Thymidine	$1.6.10^{-5}$
Glycine	3.10^{-6}

As some of these components are rather insoluble, a solution was made up at almost the final dilution and filtered (Millipore;pore size 0.22μ) into 90 ml lots to which the medium concentrate and serum were then added.

Starch gel electrophoresis of glucosephosphate isomerase (GPI)

Electrophoretic differences between the GPI enzymes in different cell lines were detected by the method of DeLorenzo and Ruddle (1969). GPI catalyses the interconversion of fructose-6-phosphate and glucose-6-phosphate. The principle of the method is the coupling of this reaction to the conversion of glucose-6-phosphate to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In the presence of the latter reaction NADP becomes reduced to NADPH which in turn, in the presence of the catalyst phenazine methosulphate, reduces the yellow soluble form of MTT. The reduced form of MTT makes an insoluble blue deposit and hence the GPI activity can be visualised.

A sample of cells was shaken with a small drop of distilled water and the resulting suspension of lysed cells was soaked up into a small strip of cellulose acetate paper (Shandon Celagram) which was then placed in a slit in a 12% starch gel (Electrostarch, Wisconsin) made up in a tris-citrate

buffer at pH 6.2. An electric potential was applied across the gel at right angles to the slit and the electrophoresis (100 volts; 30 amps; 4°C) was continued for about 4 hours. GPI activity was stained by mixing 5 ml of molten 2% aqueous agar with 5 ml of freshly prepared staining mixture and pouring this onto the gel. The gel was then incubated at 37°C and the dark blue bands representing GPI activity appeared within 30 minutes. When the required intensity of staining had been obtained the gel was fixed in methanol, acetic acid and water (mixed 2:2:3). The staining mixture was made up in tris-HCl pH 8.0. 5 ml contained the following amounts of the reagents (Sigma) for the staining reaction:

1.0 mg	NADP	nicotinamide adenine dinucleotide phosphate
1.0 mg	MTT	3(4,5 dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide
20.0 mg		fructose-6-phosphate
5λ		glucose-6-phosphate dehydrogenase (270 U/mg)
0.5 mg		phenazine methosulphate

Chromosome preparations

Cell karyotypes were determined from fixed preparations of metaphase-arrested cells. Cultures of rapidly proliferating cells were treated with Colcemid (Ciba) at a final concentration of 0.4μg/ml of culture medium for a maximum of 6 hours. The

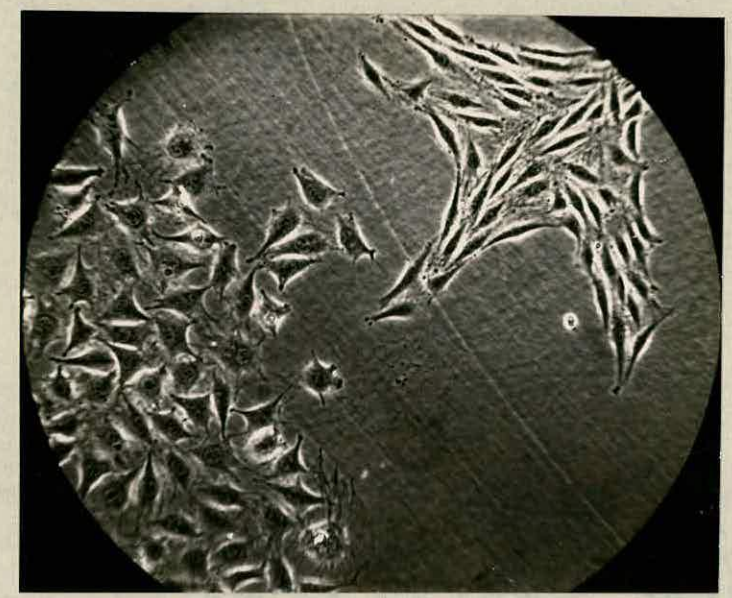

Plate 21. A clone of L_5 cells can be seen on the right. L_5 x B_{82} hybrid cells are on the left. x160

cells arrested at metaphase during that period were rinsed rapidly off the dishes with trypsin solution and collected by centrifugation (250 g 3 minutes). They were then treated with 0.075M-KCl for 8 minutes, fixed in methanol/acetic acid (mixed 3:1), dropped onto slides and stained with Giemsa.

Results and discussion

Emergence of hybrid clones

A_9 cells lack inosinic acid pyrophosphorylase activity and B_{82} cells lack thymidine kinase activity. Both these lines are therefore killed if they are cultured in HAT selection medium. When the cells had been dispersed after the monolayer fusion procedure and cultured in HAT medium, it was found that the mutant cell lines did not multiply and died off slowly but that clones of myoblasts began to appear after a few days. Occasional colonies of cells could also be seen with an unusual appearance, unlike L_5 myoblasts (see plate 21). Many clones of this sort were picked and transplanted to new dishes and were subsequently shown to be hybrid cells. Virus-induced cell fusion is not an essential part of the procedure to isolate hybrid cells. Hybrid

clones also appeared after the myoblast and L cell lines had merely been co-cultivated. Virus treatment was, however, found to increase the frequency with which the hybrid clones arose.

Transfer to ordinary medium

L_5 myoblasts were found to proliferate more slowly and to differentiate less readily in HAT than in ordinary medium. When the capacity of the hybrid cells to differentiate was under investigation they were therefore transferred to ordinary medium and at the same time clones of L_5 cells picked from the same dish were cultured in parallel to serve as controls. It has been reported that cells grown in the presence of aminopterin do not recover from its effects for some time and will not proliferate unless exogenous hypoxanthine and thymidine are present in the medium (Davidson & Ephrussi 1970). The hybrid and control cells were therefore cultured for a few days in HAT medium from which the amethopterin had been omitted, before being returned to ordinary medium. B_{82} cells died quickly in HAT medium so that when these cells were being used it was possible to pick clones and put them straight into the amethopterin-free medium. A_9 cells died more slowly and clones picked from dishes containing dying A_9 cells were therefore

cultured in HAT for a few days before transfer to amethopterin-free medium. If this precaution was not taken, sickly, floating A_9 cells, which were sometimes accidentally transferred, were able to recover. The reason for the relatively slow death of A_9 cells in HAT medium could be that cells deficient in IPP can still incorporate purines which may be released into the medium from dead cells, whereas TK deficient cells cannot incorporate thymidine from the medium. Another contributing factor may be the phenomenon of metabolic co-operation. IPP^- hamster cells have been found to incorporate radioactivity, when incubated with tritiated hypoxanthine, if they are in physical contact with cells which have IPP activity (Subak-Sharpe et al 1969). It is possible that the A_9 cells can acquire, in a similar way, either derivatives of hypoxanthine or IPP activity itself from the proliferating myoblasts in the HAT medium.

GPI phenotypes of hybrid cells

The hybrid origin of the cells which grew in HAT medium but were not of the normal L_5 myoblast morphology was confirmed by examination of their GPI phenotypes. The enzymes of the parent cell lines were found to be distinguishable by starch gel electrophoresis and hybrid cells contained

Plate 22. Electrophoresis of GPI. Samples were applied in the slits indicated by the arrow.

1 & 2. Two different myoblast x fibroblast hybrid clones.

3. Mixture of B_{82} and L_5 cells.

4. B_{82} cells.

5. L_5 cells.

activity which migrated with an intermediate mobility as well as the two parental types (see plate 22). The single intermediate band is consistent with the view (DeLorenzo & Ruddle 1969) that the enzyme is a dimer. Nine separate clones of apparently hybrid cell morphology were assayed for GPI phenotype and none of them were found to have been misclassified. This test is important because other experiments suggested that the B_{82} cells may be able to revert to HAT insensitivity (unpublished results). A similar reversion by A_9 cells has been reported (Shin et al 1973). The hybrid cells are easily distinguished by their morphology from the myoblast parent cell type, but they are less easy to distinguish from the A_9 or B_{82} cells, both of which vary very much in morphology under different conditions of culture.

Growth characteristics and appearance of hybrid cells.

A_9 and B_{82} cells proliferate more rapidly than L_5 myoblasts. The myoblast x fibroblast hybrids were also found to divide faster than their myoblast parents; after a few days of growth the hybrid clones had become distinctly larger than the L_5 myoblast clones growing in the same dish. A_9 and B_{82} cells vary in their morphology according to

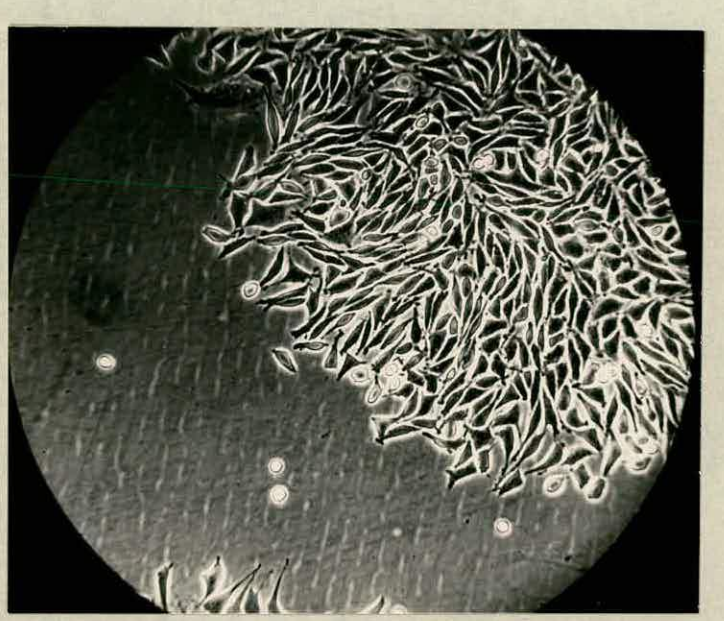

Plate 23 X 160

B82 (clone W.1)

the conditions of culture. Under conditions of rapid proliferation they become very refractile in appearance and do not adhere closely to the dish (see plate 23). L_5 cells are more adhesive under these conditions. The hybrid cells seem to inherit the adhesive characteristics of the myoblasts but have lost the characteristic bipolar shape of this parent (see plate 21).

Myotube formation

Clones of hybrid cells and of myoblasts picked from the same dishes were allowed to grow into confluent monolayers and then cultured in 2% serum. The myoblasts underwent normal differentiation, thus showing that the virus-induced fusion procedure and period in HAT medium had not damaged the cells in this respect. No myotubes were ever seen to form in the parallel cultures of hybrid cells. Occasionally syncytia were found, but these were small and similar in appearance to the rounded multinucleate cells which sometimes arise in confluent cultures of A_9 or B_{82} cells. It thus appears that the capacity to fuse into the giant, elongated syncytia, characteristic of myogenic differentiation, is extinguished in myoblast x fibroblast hybrid cells.

Chromosome complements of the hybrid cells

It has been shown that the capacity of hybrid cells to express a differentiated property characteristic of only one of the parent cell types can depend on the proportions in which the parent cells are combined (Davidson 1972). In fusion experiments involving rat and mouse cell lines hybrid cells containing two complements of chromosomes from one of the parent cell types sometimes arise (Sonnenschein et al 1971). There is evidence with the human x mouse species combination that such hybrids can arise from the doubling of one parental chromosome contribution after cell fusion (Jami and Grandchamp 1971), although triple fusions leading to viable hybrid cell lines can also take place (Ricciuti and Ruddle 1971). For the interpretation of the behaviour of a myoblast x fibroblast hybrid it is therefore important to determine the dosage contributed to the hybrid by each of the parental cell types. Chromosome preparations were made from hybrid cells and the parent cell lines 8-9 weeks after the cell fusion procedure. The chromosomes were counted in 24 metaphase spreads from each cell line and the results are shown in histogram form in figure 1. It can be seen that the hybrid cells contained, on average, rather fewer

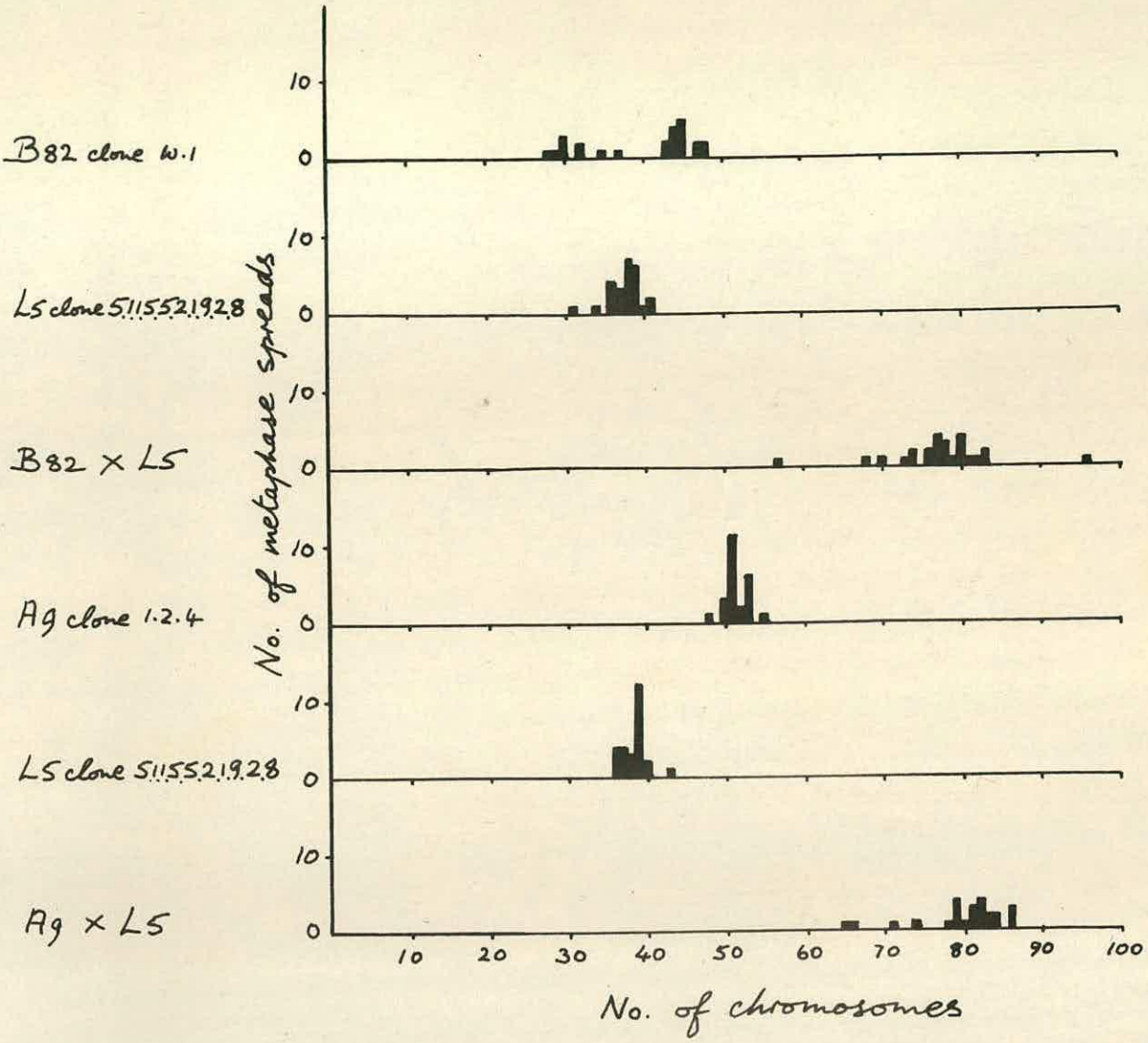
B82 clone W.1
L5 clone 5115521928
B82 × L5
Ag clone 1.2.4
L5 clone 5115521928
Ag × L5
No. of metaphase spreads
10
0
10 20 30 40 50 60 70 80 90 100
No. of chromosomes

Figure 1

chromosomes than the sum of one complement from each of the two parents. The simplest interpretation of this finding is that the hybrid cells were derived from the fusion of one L cell derivative with one myoblast and that some degree of chromosome loss took place in the succeeding 8-9 weeks.

Creatine phosphokinase

CPK activity has been shown to increase when myoblasts differentiate (Shainberg et al 1971). CPK levels were therefore assayed in cultures of proliferating hybrid cells and in hybrid cells which had been maintained in the conditions which promote differentiation by L_5 cells, that is to say, prolonged culture under confluent conditions in medium containing 2% serum. Cultures of the parent cells were assayed in parallel as controls. For both the $L_5 \times B_{82}$ and $L_5 \times A_9$ hybrids, the L_5 parental control cells were clones picked from the dishes on which the hybrid clones had first emerged. The chromosome counts of the clones were made (see above and figure 1) at the time of CPK assay.

The CPK activities of the different cells, expressed as a function of the DNA content in the samples, can be compared in the fifth column of figures in Table 3 .

TABLE 3

	No. of 9 cm dishes used for CPK assay	ΔE/min	milliunits CPK/4 dishes	μg DNA/ 4 dishes	milliunits CPK/10μg DNA
Proliferating cultures 10% serum					
B_{82} (clone W.1)	4	< .0005	< 4	30	(1.3)
L_5 (clone 5.1.1.5.5.2.1.9.2.8)	4	.0029	23.2	4	58.0
B_{82} x L_5 hybrid	4	.0070	55.0	32	17.2
'Differentiated' cultures 27 days in 2% serum					
B_{82} (clone W.1)	4	.0087	68.6	168	4.1
L_5 (clone 5.1.1.5.5.2.1.9.2.8)	2	.0388	610.6	22	277.5
B_{82} x L_5 hybrid	4 (CPK sample diluted to 1/10)	.0072	566.0	177	31.9
Proliferating cultures 10% serum					
A_9 (clone 1.2.4)	4 (CPK sample diluted to 1/10)	.0012	96	63	15.2
L_5 (clone 5.1.1.5.5.2.1.9.2.8)	4	.0087	68.6	14	49.0
A_9 x L_5 hybrid	4	.0034	27.2	5	54.4
'Differentiated' cultures 14 days in 2% serum					
A_9 (clone 1.2.4)	4	.0187	147.6	79	18.7
L_5 (clone 5.1.1.5.5.2.1.9.2.8)	2	.0301	236.8	-	-
A_9 x L_5 hybrid	4	.0110	87.0	27	32.2

CPK in L_5 cells

It can be seen from Table 3 that L_5 myoblasts undergo an increase in CPK per DNA when they differentiate. The increase is about 5 fold after 27 days in culture conditions designed to promote differentiation. This is far less than the 100 fold increases reported for L_6 (Shainberg et al 1971) and it may reflect a fundamental difference between these cell lines. However, the results in the two sets of experiments cannot be directly compared because there were a number of differences between the methods used. In the work on the L_6 cells, different culture medium was used, the cells were allowed to differentiate for a longer period and the CPK levels were measured against the total protein content in the samples instead of the DNA.

CPK in A_9 and B_{82} cells

The A_9 cells show different levels of CPK activity from the B_{82} cells. This finding is of some interest since both cell lines were derived from mouse L cells and hence are both descended from the same somatic cell. The reasons for the change in CPK level are obscure. Whether A_9 has an elevated or B_{82} a depressed level might become clear from enzyme determinations on ordinary L cells. It has been reported that other changes in

the activities of L cell sublines can occur, including the acquisition of new activities (Shodell 1972). The results in Table 3 do not show evidence of the induction of increased CPK levels in A_9 cells by prolonged culture in 2% serum. The results for B_{82} cells in Table 3 are not conclusive but other experiments failed to show an increase in CPK levels in these cells.

CPK in myoblast x fibroblast hybrid cells

Two features of the CPK levels in myoblast x fibroblast hybrid cells are of interest in the study of the expression of the myogenic differentiated state. These are first of all the base line level of the enzyme in proliferating cells and secondly the level expressed in conditions which would induce the myoblast parent cell to differentiate. The chromosome counts of the clones made at the time of the CPK assays indicated that the parental cells were near to the diploid state and that the hybrids probably arose by the fusion of one L_5 myoblast with one L cell. Since most mammalian species have about the same DNA content per cell (Ohno 1967), it is therefore probable that each parental cell contributed an approximately equal amount of DNA to the hybrid. If this was the case and if the two parental contributions acted independently in the hybrid then the level of CPK

activity per DNA in the hybrid cells should have been the average of those of the two parental cells. Any deviation from this average level is therefore evidence of interaction between the two parental contributions.

The $L_5 \times B_{82}$ hybrids showed an intermediate baseline level of CPK which was lower than the average of the parental levels. The $L_5 \times A_9$ hybrids showed a baseline level considerably higher than the parental average. These findings suggest that the two L cell derivatives have different effects on the expression of CPK by the myoblast contribution to the hybrids. This difference may be connected with the different levels of CPK which the L cell derivatives themselves express. B_{82} cells appear to exert a repressing effect on the CPK baseline in the hybrids although the enzyme activity per DNA is not as low as in the B_{82} cells. The high baseline level in the $L_5 \times A_9$ hybrids suggests a positive effect of interaction between the cell types. However, further work needs to be done to confirm these findings. High baselines of enzymes in differentiated cells have usually been found to be lowered in hybrids with cells not differentiated in that way (see Table 1 p.10)

The CPK levels found in myoblast x fibroblast hybrid cells cultured in conditions designed to

promote myogenic differentiation also show interaction between the two parental cell types. The $L_5 \times B_{82}$ hybrids showed a small increase in the enzyme activity after the period in 2% serum, and the $L_5 \times A_9$ hybrid showed a small decrease. In neither case, therefore, was a large increase in the CPK activities observed, as it was in the myoblasts cultivated in parallel. It therefore seems clear that the capacity to attain elevated CPK levels, which is characteristic of L_5 myoblasts in the culture conditions used, is extinguished in L_5 x fibroblast hybrids.

General discussion

Two conclusions about the control of myogenesis can be drawn from the present experiments, first that myotube formation is prevented in myoblast x fibroblast hybrid cells and secondly that the capacity to attain elevated levels of CPK is also extinguished in these hybrids. It is possible that the failure of myotube formation in the hybrids is a result of some non-specific damage due to the fusion process which led to the formation of the hybrid cells. It has been shown that other differentiated functions, including properties of the cell membrane, are not suppressed merely by the effects of cell fusion (see pages 11 and 15),

but it is possible to argue that myogenic cell fusion is particularly liable to interference by other sorts of cell fusion. The virus-induced formation of syncytia from myoblasts does not stimulate differentiation to occur (Chapter IV), but this does not mean that a myogenic cell could not be reconstituted after a non-myogenic cell fusion. This question could be investigated by the virus-induced fusion of proliferating myoblasts to make 2S myoblast cell lines. The isolation of products of myoblast x myoblast fusions is at present hampered by the absence of a suitable selection system. It is, however, known that the virus-induced fusion of chicken erythrocyte ghosts with myoblasts does not inhibit the subsequent myogenic cell fusion of these myoblasts (Carlsson et al 1970; see also Chapter VI). It therefore seems unlikely that virus induced cell fusion alone is sufficient to disrupt the capacity of a myoblast to differentiate.

Since cell fusion does not appear to abolish myogenesis, it is possible to interpret the failure of myoblast x fibroblast hybrids to make myotubes to mean that the mechanism which prevents myotube formation in the fibroblast acts to suppress this function in the hybrid. If this is the correct interpretation it is of interest in that it shows that a differentiated property characteristic of

the cell surface can be extinguished in cell hybrids. Previous work has shown that surface properties of neuroblastoma cells (the capacity for neurite formation and electrical excitability) can persist in neuroblastoma x B_{82} hybrid cells (Minna et al 1972). Myotube formation in myoblast x fibroblast hybrid cells could be suppressed in a number of ways and at present it is not possible to distinguish between them. It may be that it is merely the co-existence of the constituents of a fibroblast with that of a myoblast which causes the cells to fail to arrange themselves correctly for the appropriate cell to cell contacts; ordinary myogenesis is an orderly process in which myoblasts align together and fuse end to end (see plate 6). At the other end of the range of possible mechanisms it can be imagined that the fibroblast cell type specifically suppresses in the myoblast contribution to the hybrid cell, the transcription of genes concerned in the processes of myogenesis.

When myoblasts are arrested in differentiation by the prevention of cell fusion in calcium deficient medium, the CPK levels in the cultures are held in check (Shainberg et al 1969). Since myotube formation is abolished in myoblast x fibroblast hybrids it is not possible to distinguish whether their CPK levels do not show a large rise because

myotube formation is blocked, or whether the increase in CPK is under separate control in the hybrid cells.

The usual immunofluorescence procedures for the detection of actomyosin (page 58) were not suited to cultures containing multilayers of cells since thick layers of dried cells appeared to bind antibodies non-specifically. The myoblast x fibroblast hybrids formed dense multilayers when cultured in confluence conditions and so it was not possible to get clear cut results with the immunofluorescence technique. However, in view of the recent demonstration of myosin in L cells (Adelstein et al 1972), an investigation of the contractile proteins in the hybrid cells by other techniques may be worthwhile. It is not known whether myoblasts also contain small amounts of myosin (not normally detected by immunofluorescence), though recent work in this laboratory indicates that proliferating L_5 cells may do so (John and Guinness unpublished results). A comparison of the contractile proteins in L cells and in L_5 myoblasts with those found in L_5 myotubes might therefore give insight into whether the contractile proteins thought to be responsible for cytokinesis and cell movement in all sorts of cells (anon. 1971), are coded for

by the same genes as those expressed to a much greater extent in differentiated muscle cells. An investigation of the myoblast x fibroblast hybrid cells might show whether the expression of the muscle contractile proteins and of those connected with non-myogenic cell behaviour are separately controlled.

A myogenic cell line offers very interesting possibilities for work in the cell hybridisation field but enzyme deficient mutant sublines, which would be valuable in selection systems, have not yet been evolved. An attempt to make an IPP$^-$ L_5 line was made, and cells resistant to 20 μg/ml of 8-azaguanine (Koch-Light) were evolved (Klebe et al 1970a), but these cells proved able to survive in HAT medium. Some cell types possess a deaminase on the cell surface which can render certain base analogues harmless (Ruddle personal communication) and such an enzyme may have been responsible for the resistance of the L_5 cells to 8-azaguanine. Some other analogue may be more useful with these cells, though any work with the L_5 cell line which depends on continuously culturing the cells for many months is hampered by difficulties in the maintenance of the cells (see page 34).

CHAPTER VI

The expression of muscle genes in erythrocyte/muscle cell heterokaryons

Introduction

It has been shown that chicken erythrocyte nuclei can be reactivated when they are experimentally introduced into other cell types and the products of the chicken genome, inosinic acid pyrophosphorylase, cell surface antigens (Harris 1970b) and interferon (Guggenheim et al 1968)have been found in the heterokaryons. These products are characteristic of a wide range of cell types. In order to investigate whether the synthesis of a product characteristic of a specialised cell type can be evoked from chicken erythrocyte nuclei, such nuclei have been introduced into rat myoblasts and myotubes and the cultures have subsequently been monitored for the appearance of chicken muscle proteins by immunofluorescence, using species specific anti-myosin antibodies.

Materials and methods

Cell fusion procedure for the introduction of erythrocyte nuclei into myogenic cells

When chicken erythrocytes and mammalian cells are mixed together with a haemolytic concentration of Sendai virus and chilled, the erythrocytes are lysed and the erythrocyte ghosts are stuck to the

cells by adsorbed virus. If the temperature is then raised, the cytoplasm of the cells invades the ghosts and the erythrocyte nuclei are thus effectively brought into the cells (Schneeberger & Harris 1966). Erythrocyte nuclei can in this way be introduced into cells in suspension or in monolayer. The monolayer is preferable for the myoblast because this cell type has to be attached to a substratum if it is to differentiate (Jones 1964).

The procedure in a typical experiment was as follows: a 6cm dish covered with a confluent monolayer of L_5 myoblasts was rinsed three times with Dulbecco A solution. 3ml of medium without serum or antibiotics, was added and the dish was chilled on an ice-covered tray for 15 minutes. 0.1ml of a suspension of inactivated Sendai virus containing 700 HAU was then added and mixed into the medium by swirling. The dish was chilled for a further 15 minutes. 1 ml of prechilled medium containing saline-rinsed chicken erythrocytes, at a final concentration of 5-10% (v/v), was then added and mixed in. After 30 more minutes of chilling, the dish was returned to the incubator for 90 minutes and the medium and suspended cells were then thoroughly swirled, sucked off and replaced by whole growth medium containing 2% serum.

Carlsson et al (1970) have reported a similar procedure for use with primary myogenic cultures.

The technique involved adsorbing virus onto the cell monolayer and then adding the erythrocytes. The concentration of the virus still in suspension at the time that the erythrocytes were added was important. If too much was present the erythrocytes stuck together in large clumps and did not attach to the cells. It was found to be possible either to adsorb virus from a concentrated suspension onto a chilled cell monolayer and then to rinse off the surplus virus with cold medium, or to add a dose of virus just sufficient to become effectively removed from suspension by the cells. It was easier to handle several dishes at once if the latter method was used.

The fusion process was found to be more reliable with cultures of the L_5 cell line than with rat primary myogenic cultures. This may have been because newly explanted myogenic cells are less susceptible to the fusing effects of Sendai virus than are those of a cell line, as has been found to be true for other cell types (Okada and Tadokoro 1963). In addition it is probably difficult to make the virus to cell ratio repeatable in a primary culture since the ratio of myoblasts to fibroblasts is variable and virus susceptibility is cell type specific.

The chilling of the dishes is very important and was found to be reliably achieved as follows: the dishes were 'sealed' to a metal tray with a little water and this contact was not broken throughout the


MISCELLANEOUS
DISTILLED WATER
BEAKERS
CENTRIFUGE TUBES
K.W. JONES

Plate 24.

chilling procedure. Ice crystals were sprinkled over the tray which was placed on a bank of ice on top of another tray. When swirling of the dishes' contents was necessary, the bottom tray was lifted and gyrated. As the ice melted, water filled both trays and so it was necessary to suck it off continuously (see plate 24).

Erythrocytes were obtained by opening fertile hens' eggs at 10 days of incubation under sterile conditions, carefully snipping open the larger allantoic blood vessels with sharp pincers and allowing them to bleed into the allantoic fluid. This fluid was then removed by pipette, diluted in Alsever's solution, and the blood cells were collected by centrifugation. They were washed three times in Dulbecco A, the upper layer of cells in the pellet being sucked off at every centrifugation and discarded. The final preparation of washed erythrocytes was suspended in culture medium, without serum or antibiotics, and chilled in ice.

Antibodies against chicken muscle specific components

It has been shown that myosins extracted from rabbit and chicken skeletal muscle are immunologically distinguishable (Finck 1965b). Rabbit antisera prepared against chicken myosin were, however, found to stain rat myotubes very strongly when used in the indirect immune fluorescence technique (see page 58).

Plate 25. Chicken myotubes stained by the indirect immune fluorescence procedure with adsorbed anti-chicken myosin antiserum.

Plate26. L_5 myotubes stained and photographed according to the same procedures as the cells in plate 25.

It was found to be possible to adsorb out the cross reacting components in these sera (Nairn 1969) with crude saline-washed homogenates of adult rat muscle. The procedure used for removing the cross reacting components in the antisera was as follows: chilled muscle tissue was dissected from the limbs of a freshly killed adult rat, combined with an equal volume of cold Dulbecco A solution, and ground to a fine paste in the 8ml stainless steel vessel of a Sorvall Omnimixer. The paste was stirred up with several times its own volume of Dulbecco A and centrifuged at 12,000 g for 10 minutes. The supernatant was discarded and the pellet was stirred with more Dulbecco A and recentrifuged. The washing procedure was repeated until the supernatant after centrifugation was clear. 2 ml of the washed paste was then stirred with 1 ml of antiserum (see page 41), incubated at 37°C for 2 hours and then at 4°C overnight. The paste was finally removed by centrifugation (12,000 g, 10 minutes) and discarded.

After anti-chicken myosin antiserum (see page 41) had been adsorbed twice according to the above procedure and diluted to $^{1}/10$ in Dulbecco A, it was found by the immunofluorescence technique to stain chicken myotubes, prepared from chicken primary myogenic cultures, very much more strongly than those of the L_5 cell line (see plates 25 & 26). The myosin

Plate 27. x400

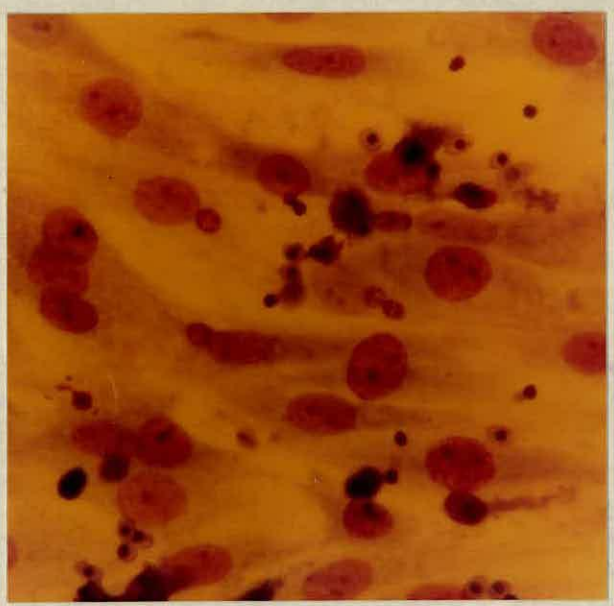

Plate 28. x400

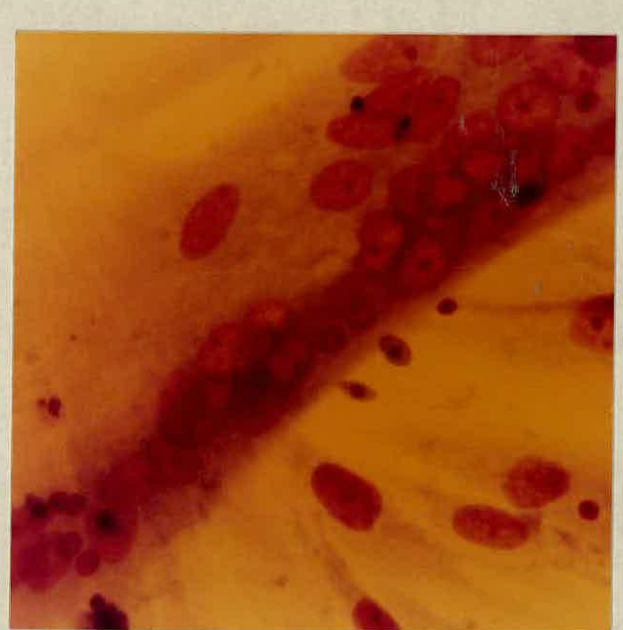

Plate 29. x400

preparation to which the antisera were prepared contained a number of components (see page 40) and the molecules with which the chicken specific antigens are associated have not been identified. The fluorescent labelling was, however, restricted to myotubes in differentiated cultures of chicken myogenic cells and this shows that the accumulation of the appropriate antigenic sites is a characteristic of differentiated muscle.

Results and Discussion

Entry of chicken erythrocyte nuclei into L_5 cells.

Dishes of mononucleate L_5 myoblasts and also of mature differentiated cultures, that had already been in 2% serum for 9-14 days, were subjected to the cell fusion procedure to introduce erythrocyte nuclei into the cells. Sample cultures of both kinds were fixed and stained (see page 49) at various intervals thereafter. Plate 27 shows cells in a mature culture, 18 hours after the fusion procedure, and plate 28 shows an undifferentiated culture that had been similarly treated. Several small nuclei can be seen in the cells. Very few nuclei of this size were found in cultures that had not been subjected to the fusion procedure. Together with the fact that the small nuclei have a similar appearance to the 'reactivated' erythrocyte nuclei in other cell types (Harris 1967), this suggests that they were of

Plate 30. L_5 myotubes dried down 14 days after chicken erythrocyte nuclei had been introduced into the cells. Stained and photographed according to the same procedures as those used for the preparations shown in plates 25 and 26.

erythrocyte origin. Plate 29 shows a differentiated culture, 18 hours after the fusion procedure. In this field, two erythrocytes that had escaped lysis by the virus can be seen near a myotube which contains erythrocyte nuclei. The contrast between the size of the erythrocyte nuclei in the myotube and in the whole erythrocytes suggests that the environment of the myoblast or myotube can induce the swelling process which is normally associated with the reactivation of these nuclei (Harris 1967).

Appearance of chicken muscle specific antigens in rat myogenic cultures

At various intervals after the introduction of the erythrocyte nuclei, sample cultures were dried and examined for the presence of chicken muscle antigens. Cells which were subjected to the full fusion procedure, but without the addition of the suspension of erythrocytes, or with the erythrocytes, but without the addition of virus, were cultured and examined in parallel as controls. The results, which were confirmed in several experiments, are shown in Table 4.

These results indicate that chicken muscle antigens are found in rat myogenic cultures, 14 days after chicken erythrocyte nuclei have been introduced into the cells. The positive fluorescence shown in the table was all found in myotubes and not in

TABLE 4

	Positive fluorescence with adsorbed antiserum	
	5 days after fusion procedure	14 days after fusion procedure
1. Mononucleate cells + virus + erythrocytes	-	+
2. Mature cultures + virus + erythrocytes	-	+
3. Mature cultures + virus	-	-
4. Mature cultures + erythrocytes	-	-
5. Syncytia made from X-rayed failed myoblasts + virus + erythrocytes		-

Plate 31. Phase contrast micrograph of myotubes 14 days after chicken erythrocyte nuclei had been introduced into the cells. x350

Plate 32. Fluorescence micrograph of the same field as that shown in plate 31; stained by the indirect immune fluorescence technique to detect chicken muscle antigens.

Comparison of plates 31 and 32 shows the presence of fluorescent and non-fluorescent myotubes.

mononucleate cells (see plates 30 & 32). Cultures never showed fluorescence in all the myotubes that they contained. The proportion of the myotubes which showed fluorescence in a positive culture varied from experiment to experiment.

In order to interpret the presence of chicken muscle antigens it is important to know the differentiated status of the host rat cells in the cultures. The treatment of mononucleate myoblasts with virus and erythrocytes did not prevent them from subsequently undergoing extensive differentiation. When examined with unadsorbed anti-myosin antiserum, cultures of this sort already showed strong positive fluorescence in the young myotubes that they contained after only 2 days in 2% serum. The sample dishes in the experimental combinations 1 to 4 can therefore all be assumed to have contained rat muscle antigens at day 5 and at day 14. Combination 5 concerns cultures of failed myoblasts (L5.1.1.5.f, see page 31), that had been given 6000r of X-rays to prevent mitosis, and then been fused into syncytia with Sendai virus by the method shown on page 56 . Previous experiments have shown that artificial syncytia constructed from failed myoblasts do not accumulate rat muscle antigens (see page 65).

Origin of the chicken muscle specific antigens

The most likely source of the chicken muscle specific antigens is the erythrocyte nuclei. Two

other possibilities must, however, be considered. The first possibility stems from the fact that the Sendai virus used was grown in the allantoic cavity of chicken embryos and the virus may have carried chicken antigens into the cultures. That the chicken muscle specific antigens did not originate in this way was shown by the lack of fluorescence in the cultures treated with virus but not with erythrocytes. The slow appearance of the antigens and the fact that they were restricted to myotubes also argues against this possibility. Another possible origin of the chicken muscle antigens, apart from the introduced erythrocyte nuclei, could be the presence of other cell types in the suspensions of erythrocytes that were used. The blood cells were repeatedly washed and centrifuged in an attempt to purify the erythrocyte population, but the possibility that small numbers of other types of cell settled among the erythrocytes cannot be completely excluded. That the appearance of the chicken muscle antigens do not result from the introduction of chicken myoblasts, which subsequently underwent natural cell fusion and differentiation, was shown by the fact that cultures treated with erythrocyte suspensions but no virus did not express the antigens.

Plate 33. Culture of L_5 cells into which chicken erythrocyte nuclei had been introduced 14 days previously. x400

Condition of erythrocyte nuclei at the time of the appearance of chicken muscle antigens

Cultures fixed and stained 14 days after the fusion procedure, at the time when the chicken muscle antigens were found, contained nuclei which resembled published photographs of erythrocyte nuclei after a prolonged period in irradiated L cells (Harris 1970b and see plate$_{33}$). Nuclear area was,however, found to be very variable in fixed preparations of differentiated cultures of L_5 cells even if the cultures had not been subjected to the fusion procedure. It was therefore not possible to be certain of the origin of individual nuclei in experimental cultures at 14 days. When chicken erythrocyte nuclei are introduced into proliferating L cells, small portions of chicken genetic material can become incorporated into some of the L cells (Schwartz et al 1971). It is conceivable that the contributions from the chicken nuclei, which were responsible for the chicken antigens in the rat myotubes, were only small portions of the chicken genome containing, for example, the myosin genes. It is also possible that full complements of the chicken genome became incorporated into rat myoblast nuclei at mitosis and entered myotubes as chicken x rat hybrid nuclei. Some erythrocyte nuclei can, however, be expected to have remained intact for 14 days. Those which could be seen inside myotubes in the mature cultures fixed

at 18 hours would presumably have remained there for the succeeding period. Furthermore, it has been shown that when the serum concentration in the medium is lowered, many L_5 cells in a culture do not undergo a round of DNA synthesis and probably not a cell division before they enter a myotube (see page 49-52). It therefore seems likely that whole erythrocyte nuclei are sometimes carried into myotubes by differentiating myoblasts. Whether it is intact erythrocyte nuclei which are responsible for the chicken muscle antigens has not been proved.

Time of appearance of chicken muscle antigens

Although chicken muscle antigens were several times found to be absent in experimental cultures at 5 days after the fusion procedure and present at 14 days, the exact time at which they can first be detected has not been conclusively demonstrated. Preliminary work suggests that the time taken for their appearance can vary from experiment to experiment. In one experiment, chicken muscle antigens were found to be present 9 days after the introduction of the erythrocyte nuclei, and in that particular experiment, the number of myotubes containing the antigens and the intensity of the fluorescence increased between cultures dried down on the 9th, the 11th and the 14th days. This suggests that chicken antigens had appeared during the 9-14 day period.

No evidence has so far been produced to indicate that the speed with which the chicken muscle antigens appear depends on the state of differentiation of the culture at the time when the erythrocyte nuclei are introduced. The experiment mentioned above, where fluorescence was found to increase in distribution and intensity during the 9-14 day period, included mature cultures, which had already been differentiated at the time of the fusion procedure, as well as cultures which had consisted of mononucleate cells, and the same result was obtained in both cases. One possible explanation of this finding is that the activation of erythrocyte nuclei takes a fixed time in any L_5 cell and that the time taken for the expression of the muscle antigens does not therefore depend on the stage of differentiation of the host cell. An alternative explanation is that erythrocyte nuclei can only be reprogrammed to myogenic activities by myoblasts that are themselves undergoing the changes in synthetic activities which normal myogenesis involves. It is probable that erythrocyte nuclei such as those shown in plate 27 are introduced directly into myotubes by the cell fusion procedure, but it is not known whether such nuclei can produce the muscle antigens. Other chicken genomes may have later been carried into myotubes by differentiating myoblasts, and it is possible that only nuclei with this history are responsible

for the chicken muscle antigens. If this is the case the evocation of muscle antigens from the erythrocyte nuclei may be an exactly comparable process in mature and in previously undifferentiated cultures in which case it would be expected to take the same length of time. Labelling experiments were performed to determine whether myoblasts in mature cultures actually do continue to differentiate. Mature cultures were fed with medium containing tritiated thymidine and after 24 hours the cultures were fixed and autoradiographs were prepared (see page 48). Labelled nuclei were found in some of the myotubes. Since intrasyncytial nuclei have with drawn from the mitotic cycle (see page 72), these nuclei must have undergone DNA synthesis and myogenic cell fusion during the period in the thymidine-containing medium. Differentiation therefore does continue in mature cultures, so it is possible that chicken muscle antigens are only evoked from erythrocyte nuclei which are introduced into myoblasts by the cell fusion procedure and subsequently become incorporated into myotubes by natural cell fusion. Further work is required to elucidate whether this is really so.

Erythrocytes from 10 day chicken embryos were chosen for the work with the L_5 myogenic cells because it has been shown that the speed of the reactivation of an erythrocyte nucleus depends on the

age of the chicken from which it came. When erythrocyte nuclei from chicken embryos at 12 days of incubation are introduced into L cells, inosinic acid pyrophosphorylase (IPP) activity derived from the chicken genes appears after 4-5 days and if younger erythrocytes are used the IPP activity appears even earlier (Harris 1970b). This is in contrast to the chicken muscle antigens which, although derived from 10 day erythrocytes, have not been detected until 9 days after the nuclei were introduced into the cells. The reason for this difference could be the general metabolic rate of the host cells. A_9, the L cell subline used by Harris, proliferates more rapidly than L_5 myogenic cells and it may be more active in other ways as well. However, IPP is an enzyme concerned in nucleic acid metabolism and hence is probably present in most, if not all, types of cell whereas muscle proteins are characteristic of a differentiated cell type. There may be basic differences in the mechanisms by which the different genes involved are activated or in the timing and rate of the expression of the gene products.

Non-appearance of chicken muscle antigens in heterokaryons made from failed myoblasts and chicken erythrocyte nuclei

Since the production of chicken muscle specific antigens can be evoked from chicken erythrocyte nuclei

Plate 34. Syncytium of failed myoblasts into which chicken erythrocyte nuclei had been introduced. Fixed 18 hours after the fusion procedure. x150

Plate 35. Similar culture to that shown in plate 34 but fixed 14 days later. x200

by the environment of a rat myogenic cell it is important to know whether this effect is a specific one. Does the effect depend on the differentiation of the host cell or can other cells which do not themselves undergo myogenesis also evoke the chicken muscle antigens? Failed myoblasts, which do not accumulate rat muscle antigens, were used as host cells for chicken erythrocyte nuclei for the investigation of this question. The line used was descended from the same clone of cells as were the cells used in the differentiating cultures and so it was considered to be the nearest available non-myogenic equivalent to them. Syncytia were made from the failed myoblasts before the erythrocyte nuclei were introduced. Plates 34 and 35 show examples of these syncytia 18 hours and 14 days after the erythrocyte fusion procedure. The erythrocyte nuclei can be distinguished from the rat nuclei at both stages by their smaller size. At 14 days the nucleoli characteristic of functionally reactivated erythrocyte nuclei (Harris 1970b) can be seen in them. As shown in Table 21 these cultures did not express the chicken muscle antigens. Similar cultures dried down at 18 days and others cultured in 10% serum for the period after the fusion procedure also proved negative in the immunofluorescence test. The failure to find chicken muscle antigens in syncytia which do not contain rat muscle antigens

suggests that the erythrocyte nuclei in the myotubes respond to specific myogenic influences in the host cells. One qualification needs to be added to this conclusion. The host cells in the failed myoblast experiment had been pretreated with X-rays to prevent cell division. When a dish of failed myoblasts is cultured for several days the cells continue to multiply, even if medium containing only 2% serum is used, and after 14 days the culture has become multilayered. Since proliferating cells often lose the genetic contributions from introduced chicken erythrocyte nuclei when they divide, a non-mitotic host cell is necessary to preserve these nuclei intact. It is conceivable that it was merely radiation damage to the failed myoblasts which prevented the evocation of muscle antigens from the introduced nuclei, although this seems unlikely in view of the fact that the pre-irradiation of fibroblast host cells does not prevent the appearance of chicken inosinic acid pyrophosphorylase or surface antigens in erythrocyte heterokaryons (Harris 1970b).

General Discussion

Evolutionary conservation of intracellular mechanisms of the control of gene activity

Interspecific crosses have often been used in the study of somatic cell genetics because they

provide more features by which the parental contributions in the hybrid can be experimentally distinguished than do intraspecific ones. Many gene products are polymorphic, even within an interbreeding population (Race and Sanger 1970), but more differences are to be found between the members of species which have diverged. The study of differential gene expression in interspecific hybrid cells and heterokaryons raises the question, however, as to whether the mechanisms of the control of gene expression in the two species from which the cells were derived are the same and can therefore interact with each other or whether they have evolutionarily diverged.

Many features of cell biology such as DNA synthesis and cell division appear to have remained unchanged throughout the animal kingdom. It might therefore be expected that the mechanisms by which these processes are controlled have also remained unchanged. It can be argued that a process such as cell division involves the interaction of many different molecules so that a change in the functional properties of one molecule would necessitate appropriate changes in the molecules with which it interacts. This would make the molecules involved in the mechanisms of cell division refractory to change by genetic drift. If the process of cell division and its control mechanisms have remained unaltered during

way the mechanisms controlling how they differentiate may also be the same. Myogenesis provides an example. This process is very similar in birds and mammals. In both cases it involves the proliferation of myogenically determined mononucleate cells, cell fusion by post-mitotic myoblasts and the accumulation of contractile proteins in elongated multinucleate synctia. Furthermore the myogenic processes have been shown to be mutually compatible within a single cell. Co-cultivated chicken and rat myoblasts have been shown to fuse into heterokaryotic myotubes (Yaffe & Feldman 1965) and chicken muscle proteins have been found in the syncytia in proportion to the number of chicken nuclei present in them (Carlsson et al 1971). It seems likely that in the common ancestor, which birds and mammals share, myogenesis took place in a way very similar to the way it occurs in modern birds and mammals and that the intracellular mechanisms by which the process is controlled have remained essentially unchanged since that time.

The comparison of intraspecific and interspecific cell hybrids made between different cell types gives further information on the evolutionary conservation of epigenetic control mechanisms. If these mechanisms have been conserved, the expression of differentiated characteristics by a hybrid between two

cell types should be the same whether the hybrid is intraspecific or interspecific. This question has not been thoroughly investigated, though the experiments which have been done so far suggest that this prediction is fulfilled for hybrids made between the cells of different mammalian species (Klebe et al 1970b; Weiss and Chaplain 1971).

The present findings with chicken erythrocyte nuclei in rat myogenic cells are of particular interest in connection with the evolutionary conservation of the mechanisms of the control of gene expression. Myotubes formed from differentiated L_5 cells, which themselves accumulate the rat muscle proteins, appear to evoke the production by chicken erythrocyte nuclei of chicken muscle specific antigens, whereas artificial syncytia made from failed myoblasts do not accumulate rat muscle proteins and do not evoke these antigens. This suggests that the erythrocyte nuclei can 'understand' the signals in the rat myogenic cells which, in normal myogenesis, promote the muscle protein syntheses directed by the rat nuclei. This is the first time that intracellular mechanisms controlling differentiation have been shown to be 'understood' across such a wide evolutionary gap as that between a chicken and a rat.

Mechanism of Activation of Muscle genes in erythrocyte nuclei

In the present state of knowledge about the mechanism of the control of gene action in eukaryotic cells, it is not possible to deduce the way in which a rat myogenic cell can signal to a chicken erythrocyte genome to produce products characteristic of a chicken muscle cell. From among the range of possible mechanisms two will serve to show how this question could be further investigated. Both models will be stated as if the erythrocyte nuclei, which are responsible for the chicken muscle antigens, are intact, although if this is not the case the principles of the models would not be affected. First of all it can be proposed that the expression of differentiated functions is controlled at the translational level. The environment of the L_5 cells may serve to activate the transcription of all the genes in an introduced chicken erythrocyte nucleus and as the cell undergoes myogenesis the translation of the muscle messenger RNA molecules may be specifically activated. Experiments on the translation of myosin mRNA *in vitro* have given evidence of translational control in myogenesis. It has been shown that muscle cells contain a ribosome-associated factor which is absent from erythroblasts and which is necessary for the initiation of myosin mRNA translation

(Rourke and Heywood 1972). Whether this factor represents the primary site of the control of muscle gene activity or is concerned, for instance, merely with the fine control of the timing of the major burst of myosin synthesis has not been determined. A model of muscle gene expression by erythrocyte nuclei involving control at the transcriptional level could be stated as follows: when the rat myogenic cell differentiates it may acquire an activator of muscle gene transcription. This activator may then enter introduced erythrocyte nuclei and specifically initiate there the transcription of chicken muscle genes. Nucleic acid hybridisation studies suggest that cells from different tissues contain different, though overlapping, spectra of RNA molecules (Church & Brown 1972), but it is not yet known whether control at the level of transcription is the principal agent in the differential expression of genes which cell differentiation involves.

Whether the expression of muscle genes by erythrocyte nuclei in myogenic cells involves control at the level of transcription or translation could be tested by the investigation of the presence of the various mRNA molecules in the heterokaryons. The technique of <u>in situ</u> hybridisation offers a means for doing this. Radioactive DNA complementary to mRNA could be prepared (Ross et al 1972), annealed

to cytological preparations and subsequently located by autoradiography (Harrison et al 1973). If both haemoglobin and myosin messenger RNA molecules were found to be present in the myogenic and non-myogenic heterokaryons, the translational model would gain support. If haemoglobin mRNA could not be detected in any of these cells and myosin mRNA only in the myogenic ones then control at a transcriptional level would seem more likely. Failure to detect an RNA molecule would not, however, prove that it is not made, since RNA molecules transcribed from genes which are not expressed at the phenotypic level may be rapidly degraded.

Expression of a differentiated activity and inherited epigenetic determination

In a complex organism such as a vertebrate it can be seen that the determination of a cell type, the inheritance of the state of determination, and the expression of the differentiated state are three distinct processes that take place at different times in the ontogeny of the organism. It has been demonstrated that when a chicken erythrocyte nucleus is placed in a myogenic cell it can express new products characteristic of the myogenic state of differentiation. This does not, however, necessarily mean that it has become determined in a new way that it can autonomously inherit. When a differ-

entiated cell such as a myoblast undergoes mitosis it is able to program both daughter cells as myoblasts. The mechanism by which it does so is one of the most important problems in developmental biology. Nuclear transplantation experiments in amphibia have demonstrated that the nucleus of a differentiated cell can be reprogrammed by the cytoplasmic environment of an egg to support the differentiation of many cell types (Gurdon & Laskey 1970; Gurdon 1972). This shows that the nuclei of somatic cells can be reprogrammed. If it can be shown whether an introduced nucleus can be reprogrammed in a heritable way by a differentiated somatic cell this would give information on the mechanism of the inheritance of the epigenetic state in that somatic cell.

The information that is so far available on this question indicates that a cell's state of determination, which is inherited by the daughter cells at mitosis, does not become transferred to separate but genetically equivalent elements within the cell. The inheritance of the epigenetic state may for instance be mediated by the process of chromosome replication so that only the direct descendants of a chromosome acquire its state of epigenetic determination. There are two lines of evidence which bear on this question, first, the study of the inheritance of the differential expression of two

alleles in a diploid cell and secondly the work on hybrid cells. It has been shown that the inactivation of a particular X chromosome in the cell of a female mammal is inherited in all the descendants of that cell (Migeon et al 1968). Allelic exclusion in antibody-producing cells represents a similar phenomenon. A single clone of antibody-producing cells expresses only one of the two alleles at a particular locus although other clones express the other allele (Weiler 1965). In both these cases two genetically equivalent elements exist in the same cell but the expression of only one of them takes place. The fact that the inactive X chromosome or the inactive immunoglobulin gene do not sometimes become activated indicates that once the state of activation or inactivation has become determined it can be inherited in an autonomous way. The second line of evidence comes from the expression of differentiated characteristics in hybrid cells. It is difficult to interpret the presence or absence of a differentiated function in a newly formed hybrid cell but if the contribution from one of the parent cells is subsequently lost, any permanent heritable change that it has induced in the other cell may become apparent. The expression of a kidney-associated esterase characteristic of a mouse adenocarcinoma, which is extinguished in adenocarcinoma

x fibroblast hybrid cells, was found to reappear when the fibroblast chromosomes were lost (Klebe et al 1970b). In similar work with hepatoma x fibroblast hybrids, tyrosine aminotransferase inducibility, characteristic of the hepatoma parent cell,but extinguished in the hybrid, was found to reappear when chromosomes were lost (Weiss & Chaplain 1971). These two pieces of work indicate that although the expression of a differentiated function may be suppressed in a hybrid cell, the contribution from the parent cell which was previously expressing this function is not necessarily reprogrammed to suppress the function for itself.

Chromosome loss from hybrid cells provides a useful means for testing whether the effect of a parental cell persists after its constituents have disappeared, but the use of species combinations where the chromosomes from one parent are systematically lost has not yet been applied to the study of the expression of a differentiated function which is retained in a hybrid cell. Can a hybrid cell which expresses a differentiated function characteristic of only one of the parental cell types continue to express this function after all the chromosomes from that parent cell have been lost? Mouse albumin is sometimes produced by hybrid cells constructed from a 2S rat hepatoma cell line and a

mouse fibroblast cell line (Peterson and Weiss 1972). This shows that the genes concerned with albumin production in the mouse cell, which were inactive in the fibroblast, have become activated in the hybrid. The relationship of the chromosome constitution of the hybrid cells to their expression of rat albumin, mouse albumin, both, or neither has not yet been worked out. It is therefore not yet known whether the mouse fibroblast can be reprogrammed to synthesise albumin in a way that it can autonomously inherit or whether it merely expresses this new differentiated function when in the presence of the hepatoma cell constituents.

The nucleus in a chicken erythrocyte is largely if not completely inactive and is post-mitotic. When it is reactivated in a different cell type, nuclear and nucleolar antigens specific to the host cell have been found in the erythrocyte nucleus and nucleolus (Ringertz et al 1971a), the nucleus resumes many activities in response to the new environment (Harris 1970b) and the experiments reported here show that it can come to express new differentiated functions characteristic of the host cell type. The erythrocyte nucleus thus comes very much under the influence of the cell into which it has been placed. If a somatic cell possesses the capacity to reprogram separate genetic elements in a way that they can autonomously inherit, it can therefore be

argued that an introduced erythrocyte nucleus would be a favourable target on which to exercise this capacity. Further work is required to devise techniques to detect such a reprogramming if it takes place. The removal of intact host nuclei by means of cytochalasin B either before or after the introduction of the erythrocyte nuclei (Ladda & Estensen 1970; Poste & Reeve 1972; Prescott et al 1972) could provide a way to distinguish between whether an erythrocyte nucleus merely comes to express differentiated functions characteristic of the host cell type or whether it can also be reprogrammed in a heritable way. As yet there are no reports in the literature of proliferating cells constructed from erythrocyte nuclei and mammalian cytoplasms. Proliferating cells would be required to prove that an epigenetic state acquired by a nucleus, as a result of cell fusion, could be autonomously inherited.

Special features of the heterokaryotic myogenic system for the study of the control of gene expression

Nuclear transplantation experiments with Xenopus have shown that the cytoplasmic environment of an egg can cause a nucleus taken from various cell types, such as an epithelial cell or a skin cell from a swimming tadpole, to support the differentiation of a range of new cell types (Gurdon & Laskey

1970; Gurdon 1972). It has also been shown that a somatic cell from a 2S clone of a rat hepatoma cell line can evoke mouse albumin production de novo, from a mouse fibroblast with which it has been hybridised (Peterson & Weiss 1972). The production of muscle specific antigens by chicken erythrocyte nuclei in the environment of rat myogenic cells provides the third example of an experimental system where the nucleus from a differentiated somatic cell can be induced to assume activities characteristic of a new differentiated state. This system provides an interesting contrast to the first two systems that were reported in three respects. First the two species from which the component parts in the hybrid system were derived are much less closely related than those in the previous work. The work with Xenopus involved different members of the same species and the work with albumin in hybrid cells involved a rat and a mouse as donor species. The chicken x rat myogenic system therefore provides more evidence on the question of the conservation, in evolution, of the mechanisms of the control of gene activity. This question could be investigated further by using nuclei from amphibian or fish erythrocytes in the rat myogenic system.

The second special feature of the myogenic system is that the myotube is a post-mitotic cell. The literature contains many references to the

Previous work on a similar system - possible reasons for the difference in result

A previous report has been made of the detection of chicken muscle specific antigens in rat myotubes into which chicken erythrocyte nuclei had been introduced (Carlsson et al 1971). However, the preliminary claim from that laboratory was retracted (Ringertz et al 1971b). Although the principles of the experiment were the same as those of the present one the results were therefore different. The reasons for this difference might be in the methods used for the detection of the antigens or in the biology of the cells.

In the methods of detection two differences seem particularly important, the fixation of the cells and the anti-chicken muscle antibody. The procedure for fixing cells used by Carlsson et al (1971) was not published but other work from their laboratory involved using an acetone:ethanol mixture to fix cells for immunofluorescence (Ringertz et al 1971a). If this fixative was used for the myogenic heterokaryon experiments it is possible that its action interfered with the particular antigenic sites by which rat and chicken muscle proteins may be distinguished. Air drying as a fixation procedure was used in the present experiments in order to interfere with antigenic sites

as little as possible, The antibody used by Ringertz et al was prepared against a crude preparation of chicken actomyosin whereas a purified preparation of myosin was used in the present experiments. This may have contained a higher concentration of the chicken muscle specific antigens, which could have led to a higher titre of the appropriate antibodies in the sera. Both these differences in the chicken muscle antigen detection procedures could have produced a difference in the sensitivity of the two methods. Carlsson et al (1971) found that myotubes formed from chicken and rat myoblasts showed positive fluorescence in their procedure but the chicken antigens may have been present in a lower concentration in heterokaryotic syncytia constructed from chicken erythrocyte nuclei and rat myogenic cells. If this were the case it could conceivably mean that a difference in the sensitivity of the two methods was the critical factor in giving different results.

The biology of the system used by Ringertz et al was different from that reported here in a number of respects. First the erythrocytes used in the present experiments were collected from chicken embryos at 10 days of incubation whereas Ringertz et al used 12-15 day embryos. Erythrocyte nuclei from younger embryos have been found to be

more quickly reactivated than are older ones in similar host cells (Harris 1970b). The population of erythrocytes in the chicken embryo also undergoes a change in kind during incubation, although the significance of this change for experiments with heterokaryons has not been explored. Primitive and definitive erythrocytes, which can be distinguished according to the type of haemoglobin that they produce, co-exist in the chick blood up to the time of hatching. Since the primitive erythroblasts do not divide after the 6th day of incubation, the proportions of the two types of erythrocyte change during the incubation period (Campbell et al 1971). If, for instance, the production of muscle antigens can only be evoked from the nuclei of primitive erythrocytes, the fall in the proportion of this type of erythrocyte between 10 days of incubation and the 12-15 day period could explain the failure of Ringertz et al to find these antigens.

The rat myogenic cells used in the two sets of experiments differ in a number of respects. Ringertz et al used newly explanted rat cells and these cells differentiate more rapidly than the L_5 cells. It is possible that the effective reactivation of the erythrocyte nucleus, known to be a slow process (Harris 1970b) is too slow, in relation to the processes of myogenic differentiation,

that many myoblasts underwent mitosis after the erythrocyte nuclei had been introduced into them. Carlsson et al observed intact erythrocytes to be carried into myotubes by post-mitotic myoblast host cells. In the mitogenic conditions that prevailed, however, a large proportion of the myoblasts observed to undergo differentiation without further cell division, may, at the time of the introduction of the erythrocyte nuclei, have already withdrawn from the mitotic cycle and undergone the myogenic maturation processes in which transitory myogenic influences may be temporally located.

There is a final possible biological reason why the L_5 cell line should evoke chicken muscle specific antigens from chicken erythrocyte nuclei whereas newly explanted rat myoblasts do not appear to do so. The intracellular processes or molecules, by which the expression of the state of differentiation is controlled in the two kinds of myoblasts, could be different either in a quantitative or a qualitative way. A quantitative difference could have arisen in the L_5 cell if it had, for instance, acquired changes in its genetic material during its long period in culture. L_5 cells show a range of 36-41 chromosomes (see page 89), compared to the rat's diploid number of 42, but this small change in overall chromosome number may encompass important rearrangements or alterations in the dosages of

the genes concerned in the control of myogenesis. Qualitative heterogeneity within the skeletal muscle myoblast cell type has not so far been demonstrated, but the fact that a cell line is normally derived from a single cultured cell means that the cell type that a line represents may not be the same as that of the majority of the cells in the original freshly explanted culture. The L_5 cell line might therefore happen to be descended from a particular kind of myoblast which had the capacity to produce certain contractile proteins not found in the majority of myotubes in a differentiated rat primary culture. The chicken equivalent to these special products might be the muscle proteins which can be identified immunologically to be of chicken origin.

By interchanging the different parts of the two systems used, it should be possible to discover which differences in method have been responsible for the difference in the results obtained by Ringertz et al and in the work reported here. The biology of the L_5 myoblast is in many ways the most special feature of the system used in the present experiments and it raises interesting possibilities for the critical differences between the two investigations. If there is some biological reason why L_5 myogenic cells can evoke the production of

chicken muscle specific antigens from chicken erythrocyte nuclei, while newly explanted rat myoblasts cannot do so, the elucidation of this reason might give important information about the mechanism of this evocation.

CHAPTER VII

Identification of nuclei on a species specific basis in myogenic cultures by in situ hybridisation

Introduction

In the study of a heterokaryotic system such as a myotube containing different kinds of nuclei, it is important to be able to identify the origin of individual nuclei. Interphase nuclei from different species can sometimes be distinguished by their different size, heterochromatic content or number of nucleoli but differences of this sort are not always available and in any case none of these criteria are very reliable - the size of a nucleus can change according to its cytoplasmic environment and the heterochromatic structures or number of nucleoli that can be resolved in the cells of one animal can also vary. The prelabelling of cells with tritiated thymidine has often been used as a method for subsequently tracing a particular population in a mixed culture but this has the disadvantage that over long periods of culture the tritium may change the behaviour of the cells into which it is incorporated. The label may also become diluted if the cells divide and there is the problem of the reutilisation, by previously unlabelled

cells, of radioactive precursors released into the culture medium from dead cells. A technique by which nuclei of different origins in fixed preparations can be differentially stained therefore has many advantages. The DNA from many higher organisms can be distinguished on a species specific basis by rapidly renaturing fractions that it contains (Hennig and Walker 1970) and the technique of *in situ* hybridisation allows specific fractions of DNA to be cytologically localised (Jones 1970). This technique has been used to trace the nuclei of mouse origin in mixed cultures of rat and mouse myogenic cells.

Materials and methods

Cell culture

Separate cultures of myogenic cells were made from the limbs of newborn rats and mice. After 2 days in culture the cells were subcultured and fractionated according to their speed of attachment to the substratum (see page 28). Part of the rat and mouse fractions containing the purified myoblast populations were mixed together and replated on new dishes and part of them were plated separately. After a few days, when differentiation had taken place, the cultures were fixed in cold methanol and acetic acid (mixed 3:1) and processed for *in situ* hybridisation.

In situ hybridisation

Radioactive RNA made *in vitro* on a template of mouse satellite DNA was annealed to fixed myogenic cultures of mouse, rat and mixed mouse/rat cells by the method of Jones and Robertson (1970). DNA in the preparations was denatured by heating because alkaline denaturation was found to remove fixed cells from the substratum on which they had been growing. The whole tissue culture dishes were dipped into boiling 0.1 SSC for 30 seconds and then plunged into ice cold 0.1 SSC, dehydrated with increasing concentrations of alcohol and dried. 2μl drops of radioactive RNA in 2 x SSC (a gift from Dr F.W.Robertson) were placed on different parts of the dishes to make several replicate hybridisation experiments. Evaporation was prevented by the use of coverslips placed over the drops and sealed to the dishes with rubber solution. The whole dishes were incubated at 65^{o}C for 20 minutes to allow the RNA to anneal to the DNA in the cells. The coverslips were then removed and the dishes were rinsed in 2 x SSC and incubated in a solution of 20μg/ml of p-RNAse (Worthington) in 2 x SSC at 37^{o}C for 20 minutes to remove single-stranded RNA. The RNAse had previously been heated at 100^{o}C for 10 minutes to remove any contaminating DNAase

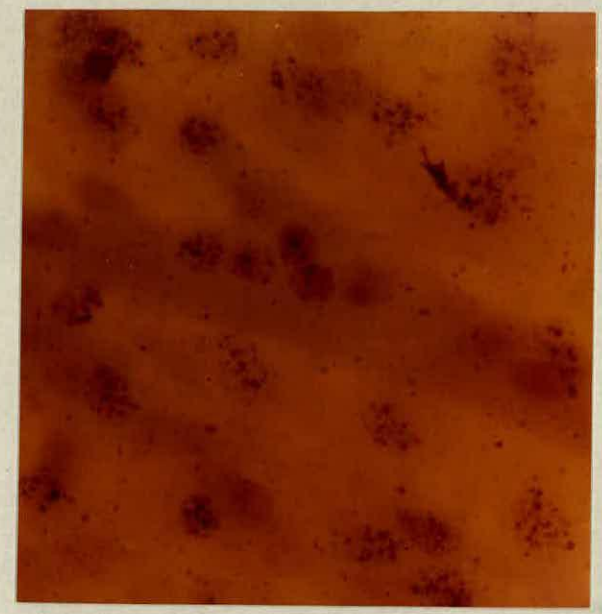

Plate 36. Mixed rat/mouse myogenic cells.
10 days exposure. x400

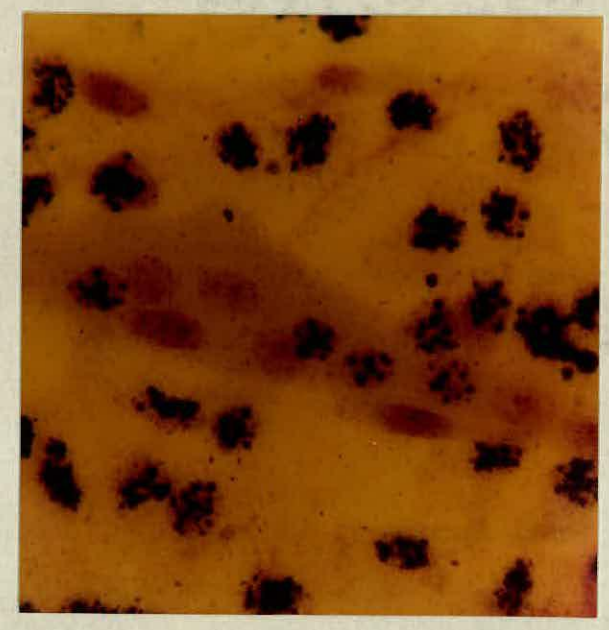

Plate 37. Mixed rat/mouse myogenic cells.
18 months exposure. x400

activity. After RNAase treatment the dishes were washed with gentle agitation in 2 litres of 2x8SC at 4°C for 4 hours to remove unbound nucleotides, dehydrated through a series of alcohols and dried. The areas of the dishes which had been subjected to the hybridisation procedure were cut out of the dishes with a hot wire and subjected to the usual procedures for autoradiography (see page 48).

Results and discussion

Autoradiographs developed after 10 days exposure showed marked labelling over the nuclei in cultures of mouse cells and only slight labelling over those in cultures of rat cells. The difference in the degree of labelling was so marked that this criterion could be used to distinguish nuclei in mixed cultures according to their specific origin. The difference became even more marked after a longer exposure time. Plate 37 shows a mixed culture of mouse and rat cells after an exposure of 18 months. It can be seen that the nuclei can be classified into two distinct groups according to the number of autoradiographic grains that they have over them.

Individual myotubes in mouse/rat mixed cultures were often found to contain both heavily labelled

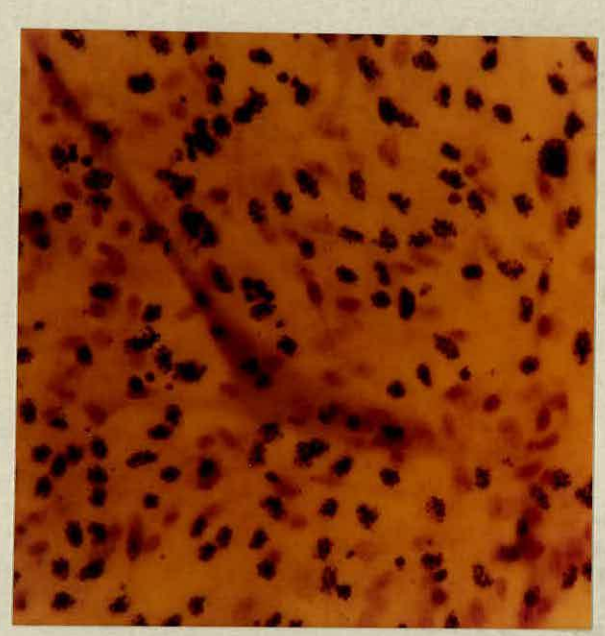

Plate 38. Mixed rat/mouse myogenic cells.
18 months exposure. x150

(mouse) and lightly labelled (rat) nuclei. Plate 38 shows an example. This myotube can be deduced to be a heterokaryon derived by the fusion of rat and mouse myoblasts. The processes by which myoblasts recognise each other can therefore act across species barriers. Rat myoblasts are also known to fuse with rabbit, calf or chicken myoblasts in culture (Yaffe and Feldman 1965).

The present experiments show that the technique of in situ hybridisation can be used to distinguish nuclei of different genetic origins in fixed preparations according to their DNA content. Mouse satellite is particularly suitable for this work both as a source of template for the synthesis of radioactive RNA because it can be isolated in large quantities from CsCl gradients and as a target for the hybridisation because its highly reiterated internal sequence allows very rapid annealing. Rapidly renaturing fractions in DNA can be isolated in other ways, such as by hydroxyapatite salt fractionation (Walker & McLaren 1965). The technique of in situ hybridisation may therefore have a wide application in the study of mixed cultures or heterokaryotic cells.

ABBREVIATIONS

CPK	creatine phosphokinase
GPI	glucosephosphate isomerase
IPP	inosinic acid pyrophosphorylase
TK	thymidine kinase
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
NADP	nicotinamide adenine dinucleotide phosphate
G1,S,G2	These letters refer to periods in the mitotic cycle of the eukaryotic cell (Howard and Pelc 1953). G1 is the period between mitosis and the period of DNA synthesis S. G2 is the period between S and the next mitosis.
1S, 2S	Because cell lines are often aneuploid it is incorrect to refer to their chromosome complements as 2N or 4N. 1S is therefore used to refer to the normal chromosome complement of a cell line and 2S to that of a cell line containing two copies of this complement.

CELL CULTURE MEDIUM & STANDARD SOLUTIONS

EAGLE'S MEDIUM - HANKS BASED	mg/litre
Inorganic salts	
NaCl	8,000
KCl	400
$CaCl_2$	140
KH_2PO_4	60
$MgSO_4.7H_2O$	200
Na_2HPO_4 (Anhydrous.)	48
Amino acids (essential)	
l-arginine HCl	125
l-cystine	24
l-histidine HCl	40
dl-isoleucine	104
dl-leucine	104
l-lysine HCl	70
dl-methionine	30
dl-phenylalanine	64
dl-threonine allo-free	96
dl-tryptophane	20
l-tyrosine	36
dl-valine	92
l-glutamine	292
Amino acids (non-essential)	
dl- serine	21
dl- glutamic acid	29.5
dl- aspartic acid	26.5

Amino acids (non-essential) cont..

	mg/litre
dl- alanine	18
l- proline	11.5
l- asparagine	15
Glycine	7.5

Vitamins

Aneurine HCl	1.0
Choline chloride	1.0
Folic acid	1.0
Inositol	2.0
Nicotinamide	1.0
Ca-pantothenate	1.0
Pyridoxal HCl	1.0
Riboflavine	0.1

Miscellaneous

Glucose	1000
Sodium pyruvate	110
Phenol red	20

HANKS' SOLUTION	mg/litre
NaCl	8,000
KCl	400
$Na_2HPO_4.2H_2O$	60
KH_2PO_4	60
$MgSO_4.7H_2O$	100
$CaCl_2$ (anhydrous)	140
Glucose	1000
$MgCl_2.6H_2O$	100
$NaH\ CO_3$	350
phenol red	10

DULBECCO A SOLUTION	
NaCl	8,000
KCl	200
Na_2HPO_4	1,150
$K_2H_2PO_4$	200
additional constituents to make Dulbecco A + B solution	
$CaCl_2$	100
$MgCl_2.6H_2O$	100

ALSEVER'S SOLUTION	
NaCl	4,200
tri-sodium citrate	8,000
glucose	2,500

adjust to pH6.1 with 10% citric acid or add 550 mg citric acid/litre.

STANDARD SALINE CITRATE (SSC)	mg/litre
NaCl	8,766
tri-sodium citrate	4,411

adjust to pH7.2 with citric acid

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